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Brit. J. Pharmacol. (1953), **8**, 259.

THE TREATMENT OF TUBERCULOSIS IN THE GUINEA-PIG WITH STREPTOMYCIN ALONE OR IN COMBINATION WITH DAPSONE, THIOSEMICARBAZONES, PAS, OR SODIUM IODIDE

BY

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(RECEIVED MAY 12, 1952)

Combinations of antituberculous drugs are usually more effective than individual drugs alone. Thus Smith, Jackson, Junge, and Bhattacharya (1949) showed that some sulphones, given in combination with streptomycin, produced a greater therapeutic effect than streptomycin alone. Moeschlin and Schreiner (1949) showed that the same was true of 4:4′-diaminodiphenylsulphone (dapsone) and *p*-aminosalicylic acid (PAS). Bloch, Vennesland, Ebert, and Gomori (1949) found that a combination of streptomycin and PAS had a much more favourable effect on experimental tuberculosis in the guinea-pig than either drug used alone. Rees and Robson (1951) studied the effect of various treatments on the cornea of mice infected with tubercle bacilli. They found that combination of PAS and *p*-ethylsulphonylbenzaldehyde thiosemicarbazone (EBT) showed no advantage over thiosemicarbazone alone. A combination of streptomycin and the thiosemicarbazone showed a definite additive effect, greater than that produced under the same experimental conditions by a combination of streptomycin and PAS. It was claimed by Woody and Avery (1948) that potassium iodide increased the antituberculous effect when given to guinea-pigs in combination with streptomycin, although Levaditi, Vaisman, and Lévy (1949), who used mice, did not confirm this observation.

In the work to be described the value of all these drugs and of *p*-acetylaminobenzaldehyde thiosemicarbazone (thiacetazone) was compared, when combined with streptomycin, under standard conditions.

METHODS

The method of intranasal instillation which produces pulmonary tuberculosis in monkeys (Francis, Spinks, and Stewart, 1950) was successfully applied to guinea-pigs. They were anaesthetized with ether, and, while lying first on one side and then on the other, had the human (Weybridge C) strain of tubercle bacilli, contained in 0.2 ml. of saline, introduced into the nostril. Previous experiments with a dye had shown that nearly all the colour passed into the lower lung when introduced in this way. In experiment 1 the dose was 10^{-4} mg. of moist bacilli grown on Löwenstein's medium for 16 days. In experiments 2 and 3 the dose of tubercle bacilli was increased to 2×10^{-4}. In every experiment treatment was begun one month after infection. During the first week of treatment in experiments 1 and 3 the guinea-pigs were given drug diet mixtures containing half the concentrations of the drugs shown in Table II, but in experiment 2 the concentrations shown were given from the start. The animals were killed one month after treatment began and examined *post mortem*. The convenient numerical assessment of lesions previously described was used (Francis, Spinks, and Stewart, 1950).

The individual scores were examined by Dr. O. L. Davies, who found that the variation in the scores between animals of the same group was greater for the higher mean scores. In order to equalize this variation it was necessary to use a square root transformation. This expedient was used in order to simplify the interpretation of the scores, although it did not alter the general trend of the results. The effect of the drugs on different organs was first added for each animal and then square roots derived. Left and right lungs, left and right bronchial lymph nodes, and the liver and spleen taken together made three groups which, added together, gave a modified score for each animal. The comparative effects of the drugs on the various organs appeared to be similar, so that the mean figure for each animal was used as a basis of assessment. Means of these scores for the three experiments are given in Table II. The differences required for significance were calculated from the variances of the modified scores within the groups of animals.

RESULTS

The actual scores of animals killed at the time treatment commenced in experiment 1 are shown in

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TABLE I

MAXIMUM POSSIBLE SCORES, AND ACTUAL MEAN SCORES OBSERVED IN CERTAIN GROUPS OF GUINEA-PIGS

Treatment	Experiment 1						
	Group I, Lung		Group II, Bronchial Lymph Nodes		Group III		Sum of Average Scores of Three Groups
	Right	Left	Right	Left	Liver	Spleen	
10 mg. streptomycin per kg. once daily, killed 2 months after infection ..	3·50	3·29	2·90	2·88	0·89	0·71	7·09
Untreated, killed 2 months after infection	4·63	5·00	2·74	2·78	1·63	1·38	9·08
Killed 1 month after infection, when treatment began	2·50	2·50	3·17	3·17	1·00	1·83	7·09
Maximum scores obtainable*	9·00	9·00	6·00	6·00	6·00	6·00	21·00

* The scores for cavitation (Francis, Spinks, and Stewart, 1950) have been omitted, because it was not observed in the present experiments and is rare in guinea-pigs.

Table I, together with the scores of animals treated for one month with streptomycin and the untreated control animals. The maximum scores which could be allocated to each organ are also shown. The results in the three experiments were similar, so Table I shows the range of the severity and the distribution of the lesions. There were six guinea-pigs per group in experiments 1 and 2 and eight in experiment 3. The results of these experiments have been combined in Table II. In experiments 1 and 3, in which a group of animals were killed when treatment began, the disease progressed in the controls but regressed in nearly all the other groups: in some groups the regression was large.

In experiment 1 dapsone, 0.05% in the food, produced the greatest increase in effect, followed closely by EBT and thiacetazone. Increasing the amount of dapsone from 0.05% to 0.3% decreased the effect, but this was restored in the presence of 0.05% sodium iodide.

In experiment 2, 0.05% dapsone again produced the best effect of any single drug given with 10 mg. streptomycin per kg. It was ineffective at a concentration of 0.01%, and in this experiment EBT 0.1% and thiacetazone 0.2% produced the next lowest effects; but thiacetazone combined with 0.05% dapsone produced the best effect, and the mixture seemed less toxic than thiacetazone alone. When given with 40 mg. streptomycin per kg. both thiacetazone and dapsone increased the effect produced by streptomycin alone, and several drugs combined with 10 mg. streptomycin per kg. produced a better effect than 40 mg. streptomycin per kg. given alone. There was a clear indication in experiment 1 that 0.05% dapsone was superior to 0.3%; in experiment 2, 0.05% dapsone again produced a good effect, but 0.01% was ineffective. In experiment 3 there was little difference in the effects produced by 0.025, 0.05, or 0.1% dapsone. In this experiment 40 mg. streptomycin per kg. produced a smaller effect than usual, and all drugs except 0.25% PAS combined with 10 mg. streptomycin produced a better effect than 40 mg. streptomycin per kg. alone. EBT 0.1% produced the best effect, but was the most toxic. In all

TABLE II

MEAN SCORES* OF LESIONS IN TREATED AND UNTREATED GROUPS OF TUBERCULOUS GUINEA-PIGS IN EXPERIMENTS 1, 2, AND 3†

Streptomycin (mg./kg.) Once Daily	Other Treatments: Percentage of Drug in Food	Mean Scores	Change in Weight per Day (g.)
10	EBT, 0·1 and 0·2‡	3·58	−1·72
10	Thiacetazone, 0·1 and 0·2 ..	4·19	+0·55
10	Thiacetazone, 0·1+PAS, 0·25 and 0·5§	3·90	−1·93
10	PAS, 1·0	3·35	−1·76
10	,, 0·5	4·35	−1·06
10	,, 0·25	5·23	−0·59
10	Dapsone, 0·01	4·45	+2·18
10	,, 0·025, 0·05, and 0·1	3·79	−0·12
10	,, 0·3	4·39	−0·22
10	Thiacetazone, 0·1 and 0·2+ dapsone, 0·05	3·69	−1·97
10	Dapsone, 0·05+PAS, 1·0, 0·5, and 0·25	3·99	−1·03
10	Sodium iodide, 0·05	4·98	+2·20
10	,, ,, 0·05+dapsone, 0·3	3·78	−0·21
10	Sodium iodide, 0·05+EBT, 0·2	3·79	+0·94
10	None	5·53	+0·54
40	Thiacetazone, 0·1 and 0·2 ..	3·66	−0·91
40	Dapsone, 0·05	3·47	−0·69
40	PAS, 0·05	3·76	−1·86
40	None	4·57	+0·84
None	Sodium iodide, 0·05	6·66	−0·97
Controls		6·86	−1·32
Animals killed one month after infection, when treatment began		5·52	

* After square root transformation.

† The difference in mean scores required for significance between groups in experiments 1 and 2 was 1.2 and in experiment 3 0.77. In preparing this Table the results of experiment 3 have been given twice the weight of either experiments 1 or 2.

‡ The average result of one group given 0.1% and another given 0.2% of EBT in addition to streptomycin.

§ The average result of one group given thiacetazone 0.1%+PAS 0.25%, and another group given 0.1% thiacetazone+PAS 0.5% in addition to streptomycin. A similar nomenclature is adopted in the rest of the Table.

experiments 10 mg. streptomycin per kg. produced a significant reduction in the extent and number of the tuberculous lesions. This effect was significantly increased by all the drugs used except sodium iodide.

When the results of the three experiments are combined (Table II) it will be seen that, except for 0.25% PAS, 0.01% dapsone, and 0.05% sodium iodide, which were probably ineffective, there was little to choose between the various drugs given in combination with streptomycin. If the scores of the lesions and the changes in weight are considered together, dapsone was the best drug when combined with 10 or 40 mg. streptomycin per kg. and PAS was inferior to EBT or thiacetazone.

Discussion

It will be seen that the maximum tolerated dose of dapsone did not produce a better effect on tuberculosis in the guinea-pig than smaller doses, and the same is true in the treatment of leprosy in man (Lowe, 1951). Sodium iodide increased the body weight of the guinea-pigs, but had no therapeutic effect, a result differing from that reported by Woody and Avery (1948) in guinea-pigs, but in accord with the findings of Levaditi, Vaisman, and Lévy (1949) in mice.

It is of interest to compare the result of treatment in guinea-pigs with that in other species. The fact that streptomycin causes regression of established lesions in guinea-pigs and man is well recognized. The author's unpublished observations show that marked regression occurs in dogs and monkeys, but macroscopic and histological examination of mice with established experimental tuberculosis indicated that although streptomycin, alone or combined with other drugs, retarded the development of lesions it did not cause regression. This is rather surprising, as the mouse is generally considered to be more resistant to tuberculosis than the monkey or guinea-pig. None of the drugs which increased the total therapeutic effect when given to guinea-pigs in combination with streptomycin produced a similar effect in tuberculous dogs.

Martin (personal communication) found that the thiosemicarbazones produced a much greater therapeutic effect on tuberculosis in mice, when given in combination with streptomycin, than did PAS, and dapsone produced little or no additive effect.

In the M.R.C. trial (Report, 1950) carried out in patients with acute progressive bilateral tuberculosis the differences between the groups treated with streptomycin alone and streptomycin plus PAS were not great. Most of them did not satisfy tests for statistical significance. The improvement was, on the whole, somewhat greater in the patients who received PAS as well as streptomycin than in those who received streptomycin alone. The big difference between the groups was, of course, in the development of streptomycin-resistance. Strains with an eightfold increase in resistance to streptomycin were isolated in 33 of 49 streptomycin cases and in only 5 of 48 streptomycin-plus-PAS cases. In two of these 5 cases there was only a single resistant culture, and deterioration in the streptomycin group was related to the emergence of streptomycin-resistance.

Streptomycin-resistance has not been observed in dogs, and it would appear that, if the three-month period of treatment in man and dogs is compared, the results in dogs are very similar to those in man, i.e., in both species the combined treatment with PAS and streptomycin produced little or no greater therapeutic effect than streptomycin alone, although this combination does produce an increased effect in mice and guinea-pigs. It seems possible, therefore, that the thiosemicarbazones may produce less additive effect in man than in mice or guinea-pigs. These drugs have been in clinical use for several years, but there seems to be no clear evidence of their value when given in combination with streptomycin. It has been claimed that the therapeutic effect in man is increased when dapsone is given in addition to streptomycin (Rist and Cottet, 1949) and that dapsone reduces the emergence of streptomycin-resistant strains (Bernard, Nouvion, Coletsos, and Kreis, 1950). On the other hand, no beneficial effect was observed by Edwards, Penman, and Cutbill (1952); moreover, toxic effects were produced, a result which is again similar to that in tuberculous dogs but different from that in guinea-pigs.

Summary

1. Human tubercle bacilli were instilled intranasally into guinea-pigs, which were one month later treated with streptomycin alone or in combination with other drugs. Treatment was continued for one month, when post-mortem examinations were performed. Most treatments caused a significant regression of lesions when these were compared with lesions in guinea-pigs killed at the time treatment began.

2. *p*-Acetylaminobenzaldehyde thiosemicarbazone (thiacetazone), *p*-ethylsulphonylbenzaldehyde thiosemicarbazone (EBT), 4:4′-diaminodiphenylsulphone (dapsone), or *p*-aminosalicylic acid (PAS) given in combination with 10 or 40 mg. streptomycin

per kg. increased the total therapeutic effect. There was little to choose between the value of these drugs at suitable doses, but dapsone produced the best effect. In two experiments the foregoing drugs, given in combination with 10 mg. streptomycin per kg., produced a greater effect than 40 mg. streptomycin per kg. given alone. Sodium iodide did not increase the total therapeutic effect when given in combination with 10 mg. streptomycin per kg.

3. These results are discussed, and it is pointed out that, although the various drugs increased the effect produced by streptomycin in guinea-pigs and mice, *p*-aminosalicylic acid produces little increased effect on pulmonary tuberculosis in man or dogs.

I am indebted to Dr. O. L. Davies for the statistical analysis and to Mr. W. Russell for technical assistance.

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Brit. J. Pharmacol. (1953), **8**, 263.

OBSERVATIONS ON ITCHING PRODUCED BY COWHAGE, AND ON THE PART PLAYED BY HISTAMINE AS A MEDIATOR OF THE ITCH SENSATION

BY

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(RECEIVED SEPTEMBER 11, 1952)

The concept of histamine as a chemical mediator of the itch sensation is not new. A substance indistinguishable from histamine exists in a combined form in the human skin and is known to be liberated when the skin is damaged in various ways (Lewis, 1927, 1942 ; Rosenthal, 1949). Lewis suggested that the itching of urticaria might be due to liberated " H " substance, and he showed that all the features of an urticarial lesion—weal, flare, and itching (" the triple response ")—could be reproduced by pricking histamine into the skin.

With such an association between histamine and itching it is not surprising that antihistamine drugs should relieve both the rash and the pruritus of urticaria. But it is unexpected that orally administered antihistamine drugs should relieve the itching of many conditions which are apparently unassociated with histamine release (for references see the Discussion).

In order to investigate this apparent anomaly it was necessary to devise some method of producing itching in normal people by some means not involving histamine. For this purpose cowhage seemed to be suitable, it being generally accepted that this produces itching in a purely mechanical fashion. But it was soon apparent that the pruritus produced by cowhage is in fact due to the liberation of histamine in the skin. It is with the demonstration of this fact, and the consideration of various consequences deriving from it, that this paper is concerned.

Materials and Methods

Cowhage is the name given to the trichomes which cover the pod of a tropical plant *Mucuna pruriens* DC. (*Dolichos pruriens* L.). At one time it gained a reputation as a therapeutic agent in the treatment of roundworm infection of the bowel (Chamberlaine, 1804). It is sometimes sold in novelty shops as " itching powder," and has been used previously for the experimental production of itching (see Rothman, 1941). A sample of the cowhage used in these experiments is illustrated in Fig. 1.

Extracts of cowhage were prepared by boiling weighed amounts of cowhage in a known volume of normal saline solution for 30 minutes. At the end of this period the fluid loss was replaced by distilled water and the trichomes removed by filtration.

" Extracted cowhage " was prepared from the boiled trichomes by boiling again with fresh distilled water for 30 minutes and filtering. The trichomes were then washed with further distilled water, and allowed to dry at room temperature.

Experiments on the Human Skin.—Comparisons of the two samples of cowhage, for instance ordinary cowhage and extracted cowhage, were made in the following way. Each subject drew in ink two circles about the size of a shilling, one on either forearm, in similar situations. The two cowhage samples, labelled " A " and " B," were applied to the marked areas of skin, one sample on the right arm, the other on the left. The arms were kept as still as possible throughout the experiment. The subjects were asked to compare subsequent sensations in respect of type, duration, and exact location (i.e., if confined to the area of application). They were also required to note the presence or absence of erythema and weal formation.

Two people applied the cowhage : one dealt only with sample " A," and the other with sample " B." The quantity of cowhage was not measured accurately but was about 2 mg. of each sample. It was applied with a metal scoop, and gently rubbed into the skin for 2–3 seconds by the index finger of the operator. Care was taken to avoid cross-contamination, and the students were warned against scratching and other ways of disseminating the cowhage. Each subject had a partner who recorded the results.

In order to test the effect of mepyramine on the itching produced by cowhage half of a group of medical students received tablets of 100 mg. mepyramine maleate t.d.s. on the day preceding, and one tablet on the morning of the experiment. The other half had control tablets of ferrous sulphate at the

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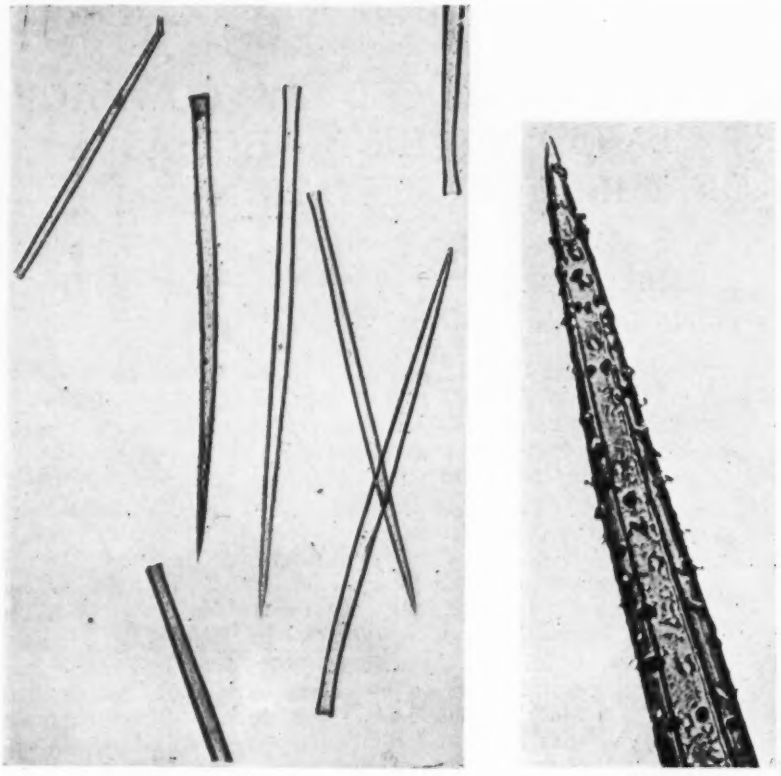

(a) (b)

FIG. 1.—Photomicrographs of cowhage. (*a*) Shows a number of hairs (trichomes) which constitute cowhage (× 46). The trichomes are pointed at one end and are between 1.5 and 2.5 mm. in length. There are approximately 450 trichomes in 1 mg. of cowhage. (*b*) A high-power view (× 300) of one trichome. The small lateral barbs, which are about 2 μ in length, are shown.

same times. The students were unaware of the nature of the tablets.

Approximately 3 hours after the ingestion of the last tablet, a cowhage-containing ointment (3.7% in paraffin) was applied to a marked area, the size of a shilling, on the forearm. This particular strength of ointment was chosen after a trial with a number of dilutions. The use of ointment is an improvement on the previous method, as it is easier to localize the cowhage. Each student was required to note the latent period between the application of the ointment and the start of the itch, the duration of the itch, and whether the itch was intense, moderate, or slight.

One week later the experiment was repeated with the two groups interchanged, so that the persons previously in the control group now had the antihistamine drug.

Throughout the paper histamine acid phosphate and morphine sulphate have been calculated as base.

RESULTS

Histamine Content of Cowhage

It has usually been assumed that both the itching and the anthelminthic properties of cowhage are mechanical effects, and that the barbs are important factors in this mechanical stimulation. While itching may certainly be produced without any evident skin reaction, many subjects show minute weals when the hairs are rubbed into the skin with a fair degree of pressure. As it is known that the nettle sting owes its properties to the presence of histamine and acetylcholine (Emmelin and Feldberg, 1947), it seemed possible that cowhage might also contain histamine. An extract obtained by boiling 1.0 g. of cowhage in 100 ml. of normal saline solution was tested for histamine (1) on the human skin, (2) on the arterial blood pressure of a cat, (3) on the isolated guinea-pig ileum, and (4) by a chemical method.

(1) 1 ml. of the extract was diluted with 9 ml. of sterile normal saline solution and 0.05 ml. of the diluted extract injected intradermally into the ventral aspect of the forearm. This resulted in the appearance of a weal and flare. The response was maximal in about 10 minutes. The weal was outlined with ink, and a permanent record obtained by a tracing on transparent paper.

In this person the weal area, 1.08 sq. cm., was the approximate equivalent of a weal produced by 1 μg. of histamine base.

A slight burning pain was noticed during the period of the injection, but there was no itching. In appearance the reaction was indistinguishable from that produced by the intradermal injection of histamine. Flushing, asthenia, and nausea were also observed, so that experiments involving the injection of extracts have been limited in number.

(2) The same afternoon samples of the extract were injected intravenously into an atropinized cat under chloralose anaesthesia (Fig. 2). No effect was produced by 0.5 ml. of the undiluted extract, but the blood pressure fell on injection of 2.5 μg. histamine. Some depression was noticed with 1.0 and 2.0 ml. of the undiluted extract and this was more prolonged than that produced by histamine.

If the weal response in the skin had been due to histamine, 1.0 ml. of the undiluted extract would have contained about 200 μg. of histamine. The effect on the blood pressure of the cat did not support this hypothesis.

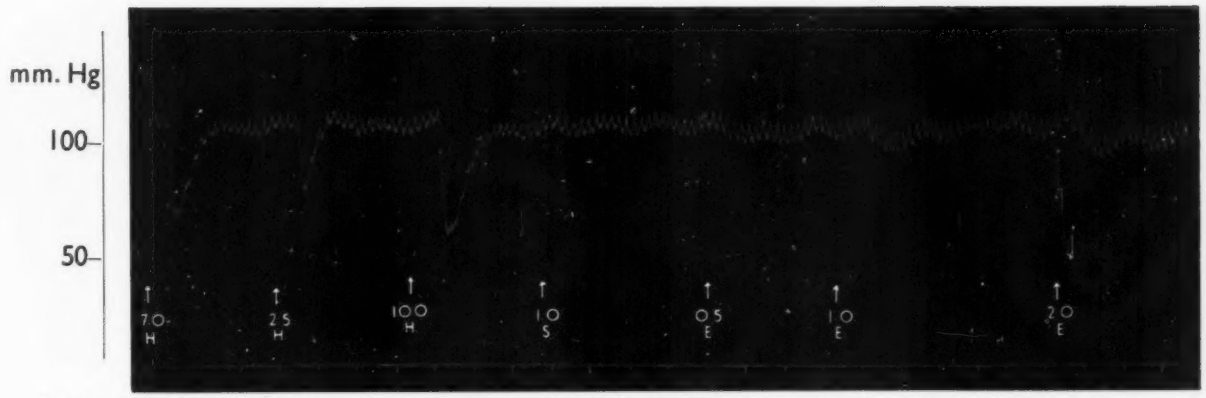

FIG. 2.—Record of arterial blood pressure of atropinized cat (2.3 kg.) under chloralose anaesthesia. Effects of intravenous injections of cowhage extract (E) in ml. and of histamine acid phosphate (H) expressed in μg. (S) 1 ml. saline solution intravenously. Time in 30 seconds.

(3) The extract caused contraction of the isolated guinea-pig ileum suspended in atropinized Tyrode solution. This contraction was compared with that produced by histamine, and it appeared that 1 ml. of extract might contain about 4 μg. of histamine, as against the expected 200 μg. per ml. suggested by the experiment on the human skin.

Thus when assayed on the human skin, the isolated ileum of the guinea-pig, and the blood pressure of a cat, the extract produced effects rather similar to histamine, but quantitatively there was no agreement.

(4) An improved colorimetric method for the chemical identification of histamine has been described by Rosenthal and Tabor (1948). Fresh extracts of cowhage were examined by this method and no histamine appeared to be present. The interpretation of the results was, however, complicated by the colour of the extracts; initially brown, they became yellow in the final solutions. An appreciable darkening of this colour could be obtained by adding 1.0 μg. of histamine base to each ml. of extract; and following the addition of 20 μg. per ml., the pink colour of histamine was unmistakable and not masked by the yellow of the cowhage.

This was taken as confirmatory evidence that histamine is not normally present in the extracts, but the observed effects might be due to the liberation of histamine from the tissues with which the extract came into contact.

Pharmacological Actions of Cowhage Extracts

Experiments on a Cat under Chloralose Anaesthesia.—Fig. 3 shows the effect on the blood pressure of an intravenous injection of an extract prepared by boiling 2 g. of cowhage in 50 ml. of normal saline solution. Both 0.5 ml. of the extract and 0.3 μg. of histamine produced a fall in the blood pressure. The latent period between the injection and the depression was about equal, but the depression due to the extract was more prolonged. Whilst the response to repeated injections of histamine remained constant, the effect of the extract increased with each dose, both in the degree and the duration of the depression. One mg. of

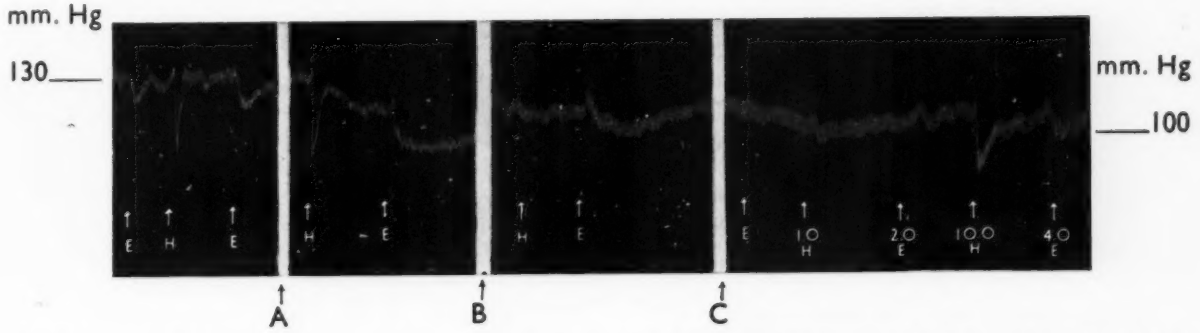

FIG. 3.—Record of arterial blood pressure of a cat under chloralose anaesthesia. Effects of intravenous injection of 0.5 ml. (E) and 4 ml. (4E) cowhage extract, and of histamine (0.3 μg., H; 1 μg., 1H; 10 μg., 10H). At A atropine 1 mg. was injected intravenously, at B and C 10 and 25 mg. mepyramine maleate respectively.

atropine sulphate intravenously did not diminish the effect of either 0.3 μg. of histamine or of 0.5 ml. extract. But after an intravenous injection of 10 mg. mepyramine maleate the effects of both histamine and extract were reduced, and after a further injection of 25 mg. mepyramine they were abolished. Larger doses of histamine (10 μg.) or of extract (4 ml.) were still active.

The depressor effect of the extracts was not abolished or diminished by bilateral vagotomy.

The Isolated Guinea-pig Intestine.—Cowhage extracts have a *p*H of about 4 ; but the contraction of the guinea-pig ileum caused by these extracts is not a *p*H effect because neutralization with sodium hydroxide, using neutral red as an indicator, did not reduce their spasmogenic properties. The addition of 1 mg. of atropine sulphate per l. of Tyrode solution likewise had no effect.

Graded doses of extract may at first produce graded responses, but repeated additions of extract to the intestine bath tend to produce less and less effect, although the sensitivity to histamine remains unaltered. Fig. 4 illustrates this point ;

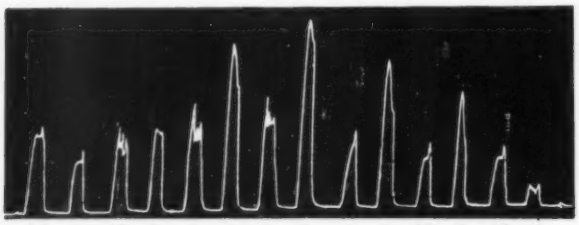

FIG. 4.—Guinea-pig ileum in 3 ml. of Tyrode at 31° C. Cowhage extract (ml.E) and histamine (μg.H) were added to the bath alternately.

whilst graded doses of histamine produce graded responses the contractions due to the cowhage extracts become gradually less.

The contraction produced by the extract is prevented or diminished by antihistamine drugs. Fig. 5 demonstrates that mepyramine maleate blocks the effect of both histamine and extract, but not that of acetylcholine. The cowhage contraction seems to be less reduced than the histamine response. The intestine still responds to 2.0 mg. of barium chloride.

The effects on guinea-pig ileum and cat blood pressure are strikingly similar to those produced by certain snake venoms and shown by Feldberg and Kellaway (1937a and b) to be due to liberation of histamine. This and the specific antagonism by mepyramine maleate suggest that the effects of cowhage extracts may also be due to histamine liberation.

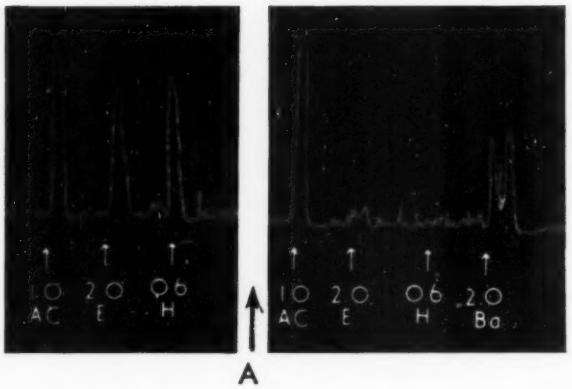

FIG. 5.—Guinea-pig ileum in 50 ml. Tyrode at 31° C. To show the effect of 1 μg. acetylcholine (A.C.), 2 ml. cowhage extract (E), 0.6 μg. histamine (H), and 2 mg. barium chloride (Ba). At A the Tyrode solution was replaced with one containing mepyramine maleate 1 mg./l.

Experiments on the Human Skin

Effect of Ordinary and Extracted Cowhage.—Table I shows that rubbing about 2 mg. ordinary cowhage into the skin produced weals in only 18 out of 26 subjects ; 3 subjects observed neither weal nor erythema, although definite itching was experienced in everyone. Wealing is uncommon with smaller quantities of cowhage, and after contamination with only a few cowhage hairs itching with only slight erythema, or no visible change in the skin, is usual.

TABLE I

A COMPARISON OF THE ITCHING PROPERTIES OF COWHAGE AND EXTRACTED COWHAGE ON 26 SUBJECTS

	Cowhage	Extracted Cowhage
Definite itching	26	3
Slight itching	0	5
No sensation	0	18
Mean duration of itching sensation	9·6 min.	1·4 min.
S.E. of the mean	±0·95	±0·69
	P<0·001	
Erythema	23	19
Weals	18	5

Preliminary experiments with dried cowhage from which extracts had been obtained suggested that its itching properties had been considerably reduced. An experiment was designed to compare the itching properties of the boiled extracted cowhage with that of ordinary cowhage. Twenty-six medical students acted as experimental subjects. The results are summarized in Table I.

With both samples a pricking sensation was reported when the hairs were being rubbed into the skin, but this is not recorded in the Table. Most subjects then experienced a latent period of

about 10 seconds, after which an itching type of sensation was noticed where the ordinary cowhage had been applied. This was usually described as an "itching sensation," but also as a "burning itch," and more rarely as a "burning pain." Other descriptions are "stinging like a nettle sting," "intense pricking," "sensation like a gnat bite," and "like prickly heat" (from a student who had lived in the tropics). The desire to scratch seemed to be universal.

At the site of application of boiled extracted cowhage the sensations described above were either absent or greatly diminished. Several subjects described an ill-defined mild itch or tickle sensation, and this is shown in the Table as "slight itching." In part, at least, this seemed to be due to movements of the arms driving the cowhage hairs further into the epidermis. Even if this sensation is not differentiated from the more intense reaction of the cowhage, it is seen that there is a significant difference between the mean durations of sensation for the two samples.

Microscopic examination showed that boiling had no appreciable effect on the morphology of the cowhage hairs, and in particular that the pointed ends of the trichomes and the small barbs were not broken by this treatment. It thus appears that the itching property is not due to the mechanical peculiarities of the cowhage hairs, but to some contained chemical substance. It is tempting to assume that this same substance is responsible for the histamine-liberating properties of the cowhage extracts.

Restoration of Itching Properties to Extracted Cowhage.—Two experiments were performed to see if it was possible to restore the itching property to the extracted cowhage by histamine and morphine. The latter was chosen because itching is a relatively common complication of morphine therapy and morphine salts are known to liberate histamine from animal tissues and also from the human skin (Nasmyth and Stewart, 1950; Feldberg and Paton, 1950).

Approximately equal quantities of extracted cowhage were rubbed into a marked area of the skin of each forearm for 3 to 4 seconds. Then 0.1 ml. saline solution was added to the cowhage on one arm and 0.1 ml. saline solution, containing either histamine acid phosphate 1 in 3,000, or morphine sulphate 1 in 500, to the cowhage on the other arm. The results are summarized in Tables II and III, and show that both histamine and morphine restore the itching property of the hairs.

As the application of histamine or morphine solutions to the unbroken skin produced no irritation, it seems that the cowhage hairs abrade the skin surface, and enable the solutions to enter the epidermis; superficial nerve endings between the cells are then stimulated by histamine to give rise to the sensation of itching. This hypothesis is supported by the observation that the substitution of fine glass wool for extracted cowhage yields similar results.

TABLE II

A COMPARISON OF THE ITCHING PROPERTIES OF EXTRACTED COWHAGE WITH NORMAL SALINE, AND WITH NORMAL SALINE CONTAINING HISTAMINE, ON 24 SUBJECTS

	0·1 ml. Normal Saline	0·1 ml. Normal Saline with 30 μg. Histamine
Definite itching	8	22
Slight itching	7	0
No sensation	9	2
Mean duration of itching sensation	3·3 min.	10·7 min.
S.E. of the mean	±0·86	±1·76
	P<0·001	
Erythema	17	24
Weals	2	23

TABLE III

A COMPARISON OF THE ITCHING PROPERTIES OF EXTRACTED COWHAGE WITH NORMAL SALINE, AND WITH NORMAL SALINE CONTAINING MORPHINE, ON 18 SUBJECTS

	0·1 ml. Normal Saline	0·1 ml. Normal Saline with 200 μg. Morphine
Definite itching	7	17
Slight itching	6	1
No sensation	5	0
Mean duration of itching sensation	4·5 min.	9·6 min.
S.E. of the mean	±1·2	±1·3
	P<0·01	
Erythema	16	18
Weals	3	17

There is considerable variation, from one experiment to another, in the duration of itching produced by the "control." This might be due to a number of factors: for example, a very severe itch on one arm might completely mask a mild sensation which would be observed were the two sensations more nearly equal in intensity.

*The Effect of Orally Administered Mepyramine Maleate on the Itch Produced by Cowhage.**—From the above observations it might be expected that an orally administered antihistamine drug would reduce the itching produced by cowhage.

A "cross-over" test was carried out in which the anti-pruritic effect of mepyramine maleate was

*This experiment was performed at University College, Ibadan, Nigeria.

compared with that of control tablets. The results are summarized in Table IV.

It is seen that the mepyramine maleate significantly prolongs the latent period before the start of the itching and decreases the duration of the itch.

TABLE IV

THE REDUCTION IN ITCHING OF A COWHAGE-CONTAINING OINTMENT BY MEPYRAMINE MALEATE, IN 13 SUBJECTS

	Control	Mepyramine Maleate 100 mg. t.d.s. by Mouth
Intense itching	13	9
Moderate itching	0	2
Slight itching	0	2
Mean latent period (in seconds) between application of ointment and start of itching ..	7·16	12·16
S.E. of mean	±1·05	±1·93
	P<0·05	
Mean duration of itching (in minutes)	6·04	4·11
S.E. of mean	±0·84	±0·32
	P<0·05	

Experiments with Histamine.—In the course of work on the relative potencies and durations of action of antihistamine drugs (Bain, Hellier, and Warin, 1948 ; Bain, Broadbent, and Warin, 1949 ; and Bain, 1949) it was necessary to make many hundreds of intradermal injections of small amounts of histamine. Whilst pain was common, even before the exhibition of a histamine antagonist, the subjects complained only very rarely of itching, and then usually with high doses, or when an injection was exceedingly superficial. The volume of the injection was 0.1 ml. or 0.05 ml., and histamine acid phosphate was present in amounts equivalent to 0.01–30.0 μg. of base, dissolved in normal saline.

Similar observations were made by Rosenthal and Sonnenschein (1946). However, Melton and Shelley (1950) describe itching following intradermal injections of histamine when, instead of being dissolved in normal saline or in distilled water, it was dissolved in the following specially buffered medium :

Sodium chloride	8.5 g.
Sodium acid phosphate	0.576 g.
Disodium hydrogen phosphate ..	2.5 g.
Distilled water to 1 litre.	

We have repeated these experiments by comparing the itching properties of histamine in normal saline and in the buffered medium of Melton and Shelley.

The volume of fluid injected was 0.1 ml., and this contained histamine acid phosphate equivalent to 2 μg. of base. These solutions were compared on 6 normal volunteers. One person experienced itching with both solutions, two with the buffered solution only. In all experiments, pain was the predominant sensation, and the itching, when present, was not intense and was of short duration (2 min., 2 min., and 1 min.) for the buffered saline solution. It therefore seems possible that when using buffered saline as the vehicle itching is more common than with normal saline. Even so itching seems to be inconstant and of short duration.

DISCUSSION

The results described above indicate that the itching properties of cowhage are not due to mechanical stimulation by the hairs, but to a chemically unidentified substance, which appears to have the property of liberating histamine from the human epidermis and dermis.

This hypothesis is supported by the following observations: (*a*) Cowhage extracts produce a triple response when injected intradermally, (*b*) the itching properties of extracted cowhage are greatly reduced, (*c*) both histamine, and morphine, which is known to liberate histamine in the skin, can restore pruritic properties to the extracted cowhage, (*d*) an orally administered antihistamine drug reduces cowhage itching.

The extracts have pharmacological effects on the cat blood pressure and isolated guinea-pig ileum resembling certain snake venoms and a substance present in bee venom (Feldberg and Kellaway, 1937a and 1937b), both of which were shown to liberate histamine from isolated animal lung, and so produced a fall in blood pressure when injected intravenously. The latent period between the intravenous injection of these venoms and the depression of blood pressure was similar to the latent period following the injection of histamine. This is also true of cowhage extracts. These three therefore differ from the histamine liberating substances described by Macintosh and Paton (1949) which have a much longer latent period.

A further difference is that the latter have no effect, except in massive doses, on the isolated guinea-pig ileum, whereas bee venom, the snake venoms, and cowhage extracts all cause a contraction. Feldberg and Kellaway believed this to be due to the liberation of histamine from the gut. With cowhage extracts the diminishing effect on repeated application, and the antagonism by mepyramine maleate, support the view that histamine liberation may be responsible. A feature common to cowhage extracts and to the organic bases of Macintosh and Paton is that progressively

larger responses, as measured on the blood pressure of the cat, follow repeated doses.

A superficial situation in the skin of the nerve endings subserving the itch sensation is suggested by the relative infrequency of itching when histamine is injected intradermally; though when applied to the epidermis—by pricking into the skin (Lewis, 1942), or by applying to scarified skin (Sollman and Pilcher, 1917), or to skin abraded by exhausted cowhage hairs—itching is usual and prominent. It is concluded that pain is the predominant sensation produced by histamine in the dermis, and that unless massive doses of histamine are injected itching is usually absent. But itching is the most prominent sensation when histamine is introduced into the epidermal layers of the skin.

In some itching conditions changes are seen mainly in the epidermis. Scabies and eczema are examples. In the lesions of prickly heat itching occurs with the early superficial retention cysts, but not with the later and more deeply situated cysts (O'Brien, 1947). In papular urticaria where the lesions are small and raised, scratching regularly provides early relief, probably by damaging the nerve endings in the papules which transmit the itch sensation.

In anaphylactic responses itching may occur as the first or only symptom (Klinkert, 1923): and either generalized pruritus (Nomland, 1944) or a generalized pruritic urticaria may result from the injection of acetyl-β-methyl choline into people who respond in this way to effects of heat, exertion, or emotion (Grant, Comeau, and Pearson, 1936). Further evidence is provided by the present experiments with cowhage, which unless rubbed hard into the skin does not produce bleeding points. Itching, however, is always observed and is practically the sole effect when small quantities come in contact with the skin. It seems, therefore, that the trichomes remain in the epidermis. Moreover, as cowhage produces little or no itching where the keratinized layer of the epidermis is thick, as in the palm of the hand, the nerve endings are probably situated in the basal layers of the epidermis. All these observations agree with those of Torok (1907) and Kenedy (1936), who noted that itching cannot be elicited from areas of skin denuded of epidermis.

In urticaria the main changes are seen in the dermis, but itching, which is usually marked, precedes the appearance of the weal. In this respect it differs from the lesions of dermographism in which itching is usually, but not always, absent. Both are believed to be due to abnormal histamine release, as they resemble in appearance the triple response to histamine, and they are both controlled by antihistamine drugs (Bain, Hellier, and Warin, 1948). The lesions of urticaria can be mimicked by applying histamine to the abraded epidermis. It is tempting to postulate that in urticaria some, at least, of the histamine is liberated amongst the basal epidermal cells, whereas in dermographism mechanical stimulation of the skin causes liberation only in the dermis.

The precise relationship between histamine release and itching requires further consideration. It is well known that the systemic administration of histamine-liberating drugs, such as morphine and stilbamidine (Macintosh and Paton, 1949), produces itching in a proportion of patients, and that morphine will exacerbate an existing itch. Also, drug rashes which follow the administration of a drug to a sensitive patient are frequently pruritic. However, the intravenous injection of histamine in man does not cause pruritus (Weiss, Robb, and Ellis, 1932). It is therefore likely that the itching in drug hypersensitivity reactions, or after the systemic use of a histamine liberator, is due to histamine liberation in the basal epidermal layers of the skin. It is not to be expected that histamine liberated from other body tissues will cause itching.

The objection that all itching drug hypersensitivity reactions are not associated with a triple response can be met, as it seems that the release of sufficient histamine in the epidermis to cause severe itching may not be accompanied by a triple response, which is a dermal reaction. This view, which is in opposition to the contention of Rothman (1941) that itching without visible changes in the skin cannot be due to histamine, is strengthened by many reports that orally administered antihistamine drugs have some effect in relieving the pruritus of such conditions as eczema, prickly heat, chickenpox rashes, mosquito bites, the dermatitis of poison ivy, pruritus ani and vulvae (of uncertain aetiology), and pruritus associated with Hodgkin's disease, and obstructive jaundice (Baer and Sulzberger, 1946; Blumenthal and Rosenberg, 1947; Davis, 1950; Feinberg, 1950; Friedlaender and Feinberg, 1946; Gordon and Crewe, 1948; Hunter and Dunlop, 1948; Kuhn, 1949; Lynch, 1947; Silverman, 1949; Waldbott, 1946; and Waldriff, Davis, and Lewis, 1950). In many instances improvement in the pruritus leaves the rash unchanged.

In many itching conditions there is evidence that antigen-antibody mechanisms are involved, and in these circumstances one might expect the liberation of histamine. Drug rashes have already

been mentioned. In scabies the itching starts only when the patients become sensitive to some product of the causal mite (Mellanby, 1943); similar considerations apply to the itching of mosquito bites (Mellanby, 1946; Gordon and Crewe, 1948; Heilesen, 1949) and possibly to the itching lesions associated with other types of "insect" bite.

An antigen-antibody reaction is also well established in contact dermatitis. But it must be admitted that in many itching rashes the precise mechanism of cellular damage, sufficient to liberate histamine, but not so severe as to destroy the superficial nerve endings, remains unknown. Moreover, in the generalized pruritus of toxic conditions such as chronic nephritis, diabetes, and obstructive jaundice, there is no direct evidence to implicate histamine. But in all these conditions there are gross disturbances of the blood chemistry, and it may be that some metabolite has either histamine-liberating properties or may itself act as an antigen. In any event it seems certain that the liberation of histamine in the epidermis causes itching, and the fact that such itching may occur without any visible vascular or other effect in the skin makes it impossible to argue that when itching is not associated with vascular effects it cannot be due to histamine.

Summary

1. The itching produced by cowhage is not a mechanical effect, but is due to a chemically unidentified substance which liberates histamine in the human epidermis.

2. In its pharmacological effects this substance resembles others present in some snake venoms, and also in bee venom, which have been shown to liberate histamine from animal tissues.

3. The nerve endings which subserve itching are located in the basal layers of the epidermis, and are probably absent from the dermis.

4. Histamine liberation in the epidermis can produce marked itching unassociated with a triple response.

5. It is probable that histamine release is involved in many more itching conditions than has been hitherto supposed.

Grateful acknowledgments are due to the various volunteers, both British and Nigerian; to Dr. G. Achari for performing the colorimetric tests for histamine; to Mr. G. A. Nelson, who has kindly supplied the slides from which the microphotographs were prepared, and the information about the dimensions and weight of the trichomes; and especially to Professor W. A. Bain for suggestions, encouragement, and much helpful criticism.

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TESTOSTERONE PHENYL PROPIONATE (TPP): BIOLOGICAL TRIALS WITH A NEW ANDROGEN

BY

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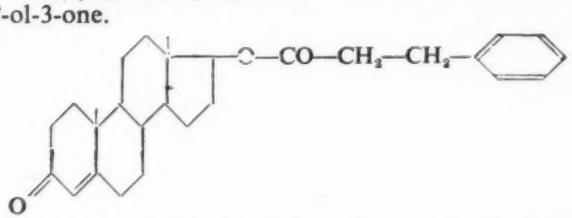
Testosterone propionate (TP) has been for a long time the most effective androgenic substance available. The search for a more potent compound has not, however, been allowed to lapse, as the need for such a therapeutic agent has been apparent for some years; during this time a number of highly active and easily made oestrogens have been discovered, and it has been hoped that parallel advances would widen the field of use of the male sex hormones. So far no synthetic non-steroid substance has been found to have marked androgenic activity. Attempts to increase the potency and duration of action of testosterone compounds have been limited to efforts to delay the absorption by forming crystalline depots or by adsorbing the androgen on aluminium phosphate, but these have not been uniformly successful. It was not until 1951 that a more potent compound, testosterone cyclopentylpropionate (TCP) was found by Ott, Kuizenga, Lyster, and Johnson (1952) and first reported by Sakamoto, Gordan, and Eisenberg (1951) and Lloyd and Fredericks (1951).

At the same time in these laboratories, Dr. C. L. Hewett (1951) produced another compound with a long side chain, testosterone phenyl propionate (TPP), which is easily made, and as far as can be ascertained has not been mentioned previously in the literature. This ester occurs as colourless needles melting at 116.5° C., $[\alpha]_D^{20°} + 88.7°$, and is readily soluble in the usual solvents. Chemically it is the β-phenyl-propionate of Δ^4-androsten-17 β-ol-3-one.



It was decided to investigate the maximal effect and duration of effect of single doses of TPP as compared with TP, and also to find the most suitable medium for injection of TPP and the most effective route of administration. A study of the morphological changes resulting from administration of TPP was also undertaken, with particular attention to any toxic effects from excess dosage.

METHODS

Assay Methods.—Normal immature 40- to 50-g. male albino rats from Wistar stock were used while being fed on Aberdeen Standard diet. The animals were castrated through a scrotal incision, and were injected 14 days after castration. Each animal received a single injection either subcutaneously in the back or intramuscularly in the hind leg. At intervals after the injection, groups of 3 to 6 animals were killed and weighed. The seminal vesicles and prostate were then dissected out, by cutting through the vasa deferentia and the urethra near the base of the bladder; the coagulating glands were then teased from the seminal vesicles, which were cut off as near as possible to the ejaculating ducts, and weighed, while fresh, on a torsion balance to the nearest 0.1 mg. The prostate and urethra were fixed overnight in Bouin's fluid, and on the next day the urethra was removed and the whole prostate dried on blotting-paper and weighed to the nearest 0.1 mg.

In all these experiments 1,012 male rats were used.

The preliminary tests were carried out by the method of Mathieson and Hays (1945), using subcutaneous injections of testosterone propionate (TP) in sesame oil as a standard of known androgenic activity whose effects were compared with those of TPP in sesame oil at two dose levels, 0.2 mg. and 0.8 mg., both in 0.2 ml., the ratio between the two dose levels being 1:4, and the lower dose being near the minimum for obtaining a measurable response. Other vehicles for the two androgens were also compared at these dose levels, by both intramuscular and subcutaneous routes.

In the second series of experiments a higher dose of 2.5 mg. in 0.1 ml. was used throughout, and readings were taken over a longer period to investigate the rate of involution of the glands. Readings were also made at very short intervals after injection, to

examine more fully the rate of growth to the peak. In these experiments TPP was again compared with TP, and it was also desired to compare the effects of different solvents and different routes of injection.

Control groups of castrated animals were untreated or injected intramuscularly with placebo solvents (ethyl oleate, sesame oil, and blank emulsion). These were killed on the 3rd and 10th days after injection.

The preliminary experiment carried out at two dose levels was treated as a 4-point test on the assumption that TPP and TP had qualitatively similar effects. The response at 5 days after injection was arbitrarily selected for the comparison.

It was felt, however, that none of the usual methods of comparison would give a clear expression of relative potencies, since both intensity and duration of activity were involved; it was possible to compare these independently, but preferable to find a single expression of potency. Accordingly, the weight of glands was plotted against time, and the area enclosed by each graph up to the point of 50% involution from peak stimulation, using the control level as ordinate, was used as an expression of the potency. The activity-duration index arrived at is in "milligram-day" units (from the graphical axes). This can readily be expressed as a ratio to the effect of TP in sesame oil, which was taken as unity at each dose level.

The activity-duration index estimated from the graphs in "milligram-day" units is included in the tables, except where no normal growth-time curve could be obtained, either through too little stimulation (doses of 0.2 mg.) or through irregular results (as were obtained through using the supersaturated solution of TPP in propylene glycol). The activity-duration as a ratio to TP in sesame oil as unity (at each dose level) is also shown in the tables.

Histological Examination. — The morphological effect of TPP on the testes in immature rats was studied by comparison of 12 treated animals with 12 untreated controls, the animals being of about 45 g. body weight. The treated animals were injected subcutaneously with 0.8 mg. of TPP in 0.2 ml. of sesame oil, and 3 of each group were killed on the 3rd, 4th, 7th, and 14th days after injection, and the testes completely sectioned and microscopically examined.

Toxicity tests were carried out by gross and microscopic examination of various organs in rabbits of both sexes. Six young rabbits (litter-mates) weighing approximately 1,300 g. were used. Four of these (2 male, 2 female) were injected subcutaneously with 30.5 mg. of TPP in 0.5 ml. sesame oil three times weekly for four weeks, the total dose of 366 mg. per rabbit being equivalent in testosterone content to 300 mg. of testosterone propionate; two rabbits (1 male, 1 female) served as controls. Body weights were recorded once weekly, and on the 30th day all animals were killed for histopathology. Tissues were fixed in Bouin's fluid and paraffin slices about 5 μ in thickness were cut. The haemalum-eosin method was used for general purposes. Bone-marrow was fixed in Zenker-formol solution, smears of bone-marrow being stained by Leishman's stain; for bone-marrow sections haemalum-eosin, haematoxylin-azo eosin, and eosin-methylene blue methods were used, some sections from each animal by each method.

Drugs and Media

Subcutaneously: Preliminary experiments: TP and TPP in sesame oil and in emulsions prepared by the method of Lens, Overbeek, and Polderman (1949) (all 1 mg./ml. and 4 mg./ml.). Higher-dosage experiment: TPP in ethyl oleate, propylene glycol, and aqueous suspension (all 25 mg./ml.). Histological experiment: TPP in sesame oil (4 mg./ml. for rats; 61 mg./ml. for rabbits).

Intramuscularly: Preliminary experiments: TP and TPP emulsions (1 mg./ml. and 4 mg./ml.). TP aqueous suspension (BDH) (5 mg./ml.). Higher-dosage experiment: TP and TPP in sesame oil, ethyl oleate, and emulsion (all 25 mg./ml.). TPP in propylene glycol (25 mg./ml. supersaturated). TP aqueous suspension (BDH) and TPP aqueous suspension (both 25 mg./ml.).

TABLE I

PRELIMINARY EXPERIMENT

Mean responses ± S.E. (3–6 rats) of seminal vesicles and prostates of castrated rats to doses of 0·2 mg. and 0·8 mg. of TP and TPP given subcutaneously in 0·2 ml. sesame oil

Days after Injection	Testosterone Propionate				Testosterone Phenyl Propionate			
	0·2 mg.		0·8 mg.		0·2 mg.		0·8 mg.	
	Sem. Ves. (mg.)	Prostates (mg.)	Sem. Ves. (mg.)	Prostates (mg.)	Sem. Ves. (mg.)	Prostates (mg.)	Sem. Ves. (mg.)	Prostates (mg.)
3	17·0±2·8	31·6±3·1	22·0±3·7	42·0±6·6	17·3±2·5	30·0±6·1	24·4±4·1	49·8±4·2
4	19·6±1·8	32·3±3·7	*31·5±3·8	47·0±6·1	25·5±3·9	37·5±4·0	34·8±9·6	51·5±14·0
5	18·8±5·8	37·0±5·8	31·1±4·5	49·7±11·9	26·7±4·2	43·7±7·9	45·6±10·0	73·3±11·1
7	—	—	26·0±14·2	*55·1±14·3	—	—	*60·5±12·4	*118·8±14·4
14	—	—	15·5±1·3	33·0±10·7	—	—	49·0±14·7	90·8±9·5
21	—	—	13·0±4·8	24·5±8·0	—	—	25·8±4·4	48·0±16·0
28	—	—	13·0±1·0	17·5±0	—	—	24·3±3·2	27·7±4·3
Activity-duration Index	—	—	250	575	—	—	700	1,400
Activity-duration Ratio	—	—	1·0 (Standard)	1·0 (Standard)	—	—	2·8	2·4

* Peaks.

Results

Comparative Tests.—The most important of the results from the preliminary experiments on rat seminal vesicle and prostate growth are given in Table I, from which Figs. 1 and 2 have been constructed.

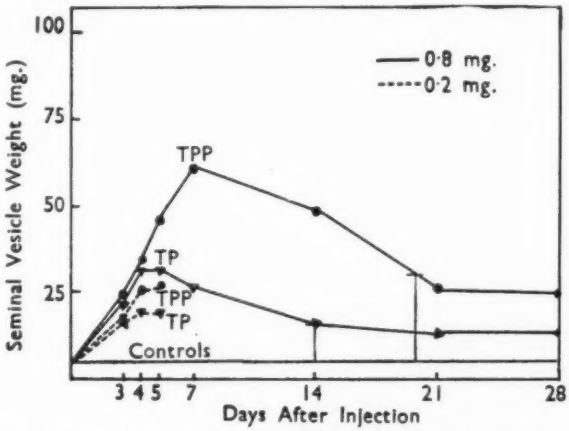


FIG. 1.—Effect on seminal vesicle weight of doses of 0.2 mg. and 0.8 mg. of TP and TPP in sesame oil injected subcutaneously in castrated rats. Vertical lines are drawn through the points of 50% involution from the peak readings to give the areas taken as the activity-duration indices.

It was found, by applying 4-point procedure to the readings at 5 days for TP and TPP given to rats subcutaneously in sesame oil in doses of 0.2 mg. and 0.8 mg., that TPP was about 2.7 times as potent an androgen as TP, the result being the same for both seminal vesicles and prostates. On the other hand, when the activity-duration index was adopted for this experiment the potency of TPP was found to be about 2.8 (seminal vesicles) and 2.4 (prostates) times that of TP. None of the emulsions or suspensions tried at these dose levels was as potent as TP in sesame oil.

From the results of the second series of experiments the activity-duration indices and ratios have been calculated (Table II), and Figs. 3 and 4 have been constructed.

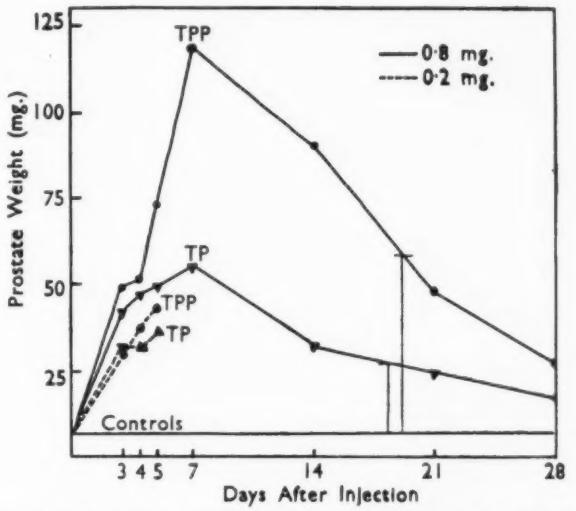


FIG. 2.—Effect on prostate weight of doses of 0.2 mg. and 0.8 mg. of TP and TPP in sesame oil injected subcutaneously in castrated rats. Vertical lines are drawn through the points of 50% involution from the peak readings, as in Fig. 1.

The mean control values for seminal vesicles varied from 3.7 mg. to 5.7 mg. and for prostates from 5.3 mg. to 10.8 mg., none of the groups injected with placebo solvents showing any significantly different results from the untreated group.

The figures obtained from the activity-duration indices showed that in sesame oil TPP was about 3.2 (seminal vesicles) and 4.8 (prostates) times as potent as TP, and in ethyl oleate TPP was about 4.2 (seminal vesicles) and 4.4 (prostates) times as potent as TP, when given to rats intramuscularly at a dose of 2.5 mg./0.1 ml.

Ethyl oleate was about 1.6 (seminal vesicles) and 1.6 (prostates) times as effective as sesame oil as a solvent for TPP when used intramuscularly and about 2.3 (seminal vesicles) and 2.1 (prostates)

TABLE II

HIGHER-DOSAGE EXPERIMENT

Activity-duration indices and ratios based on response/time graphs from mean responses (5 rats) of seminal vesicles and prostates of castrated rats to a dose of 2·5 mg. of TP and TPP in 0·1 ml. Groups killed at 8 hrs. and 1, 3, 5, 7, 9, 11, 14, 21, 28, 35, and 42 days after injection

Androgen	Medium	Route of Injection	Activity-duration Index		Activity-duration Ratio	
			Seminal Vesicles	Prostate	Seminal Vesicles	Prostate
TP	Sesame oil	Intramuscular	500	425	1·0 (Standard)	1·0 (Standard)
TPP	,, ,,	,,	1,575	2,025	3·2	4·8
TP	Ethyl oleate	,,	625	725	1·25	1·7
TPP	,, ,,	,,	2,600	3,175	5·2	7·5
TPP	,, ,,	Subcutaneous	3,950	4,225	7·9	9·9
TP	Aqueous emulsion	Intramuscular	500	400	1·0	0·9
TPP	,, ,,	,,	750	675	1·5	1·6
TP	Aqueous suspension	,,	450	475	0·9	1·1
TP	,, ,,	Subcutaneous	850	725	1·7	1·7
TPP	,, ,,	Intramuscular	575	500	1·2	1·2
TPP	,, ,,	Subcutaneous	1,700	1,475	3·4	3·5

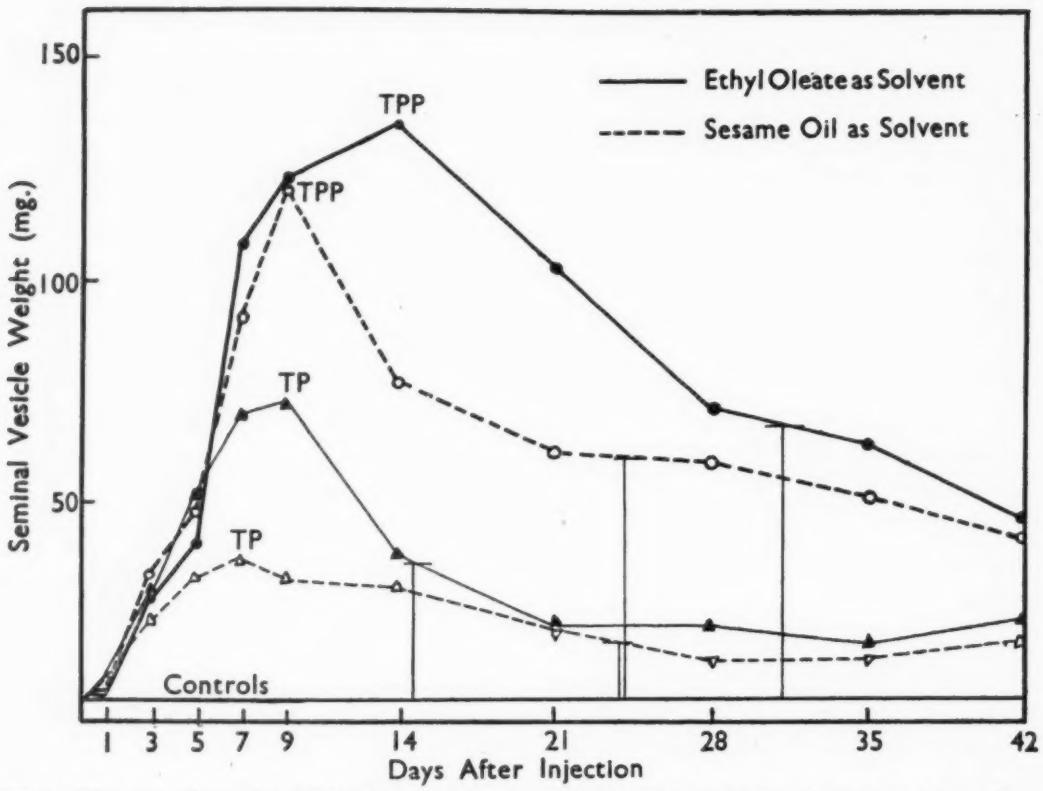


FIG. 3.—Effect on seminal vesicle weight of doses of 2.5 mg. of TP and TPP in sesame oil and ethyl oleate injected intramuscularly in castrated rats. Vertical lines as in Fig. 1.

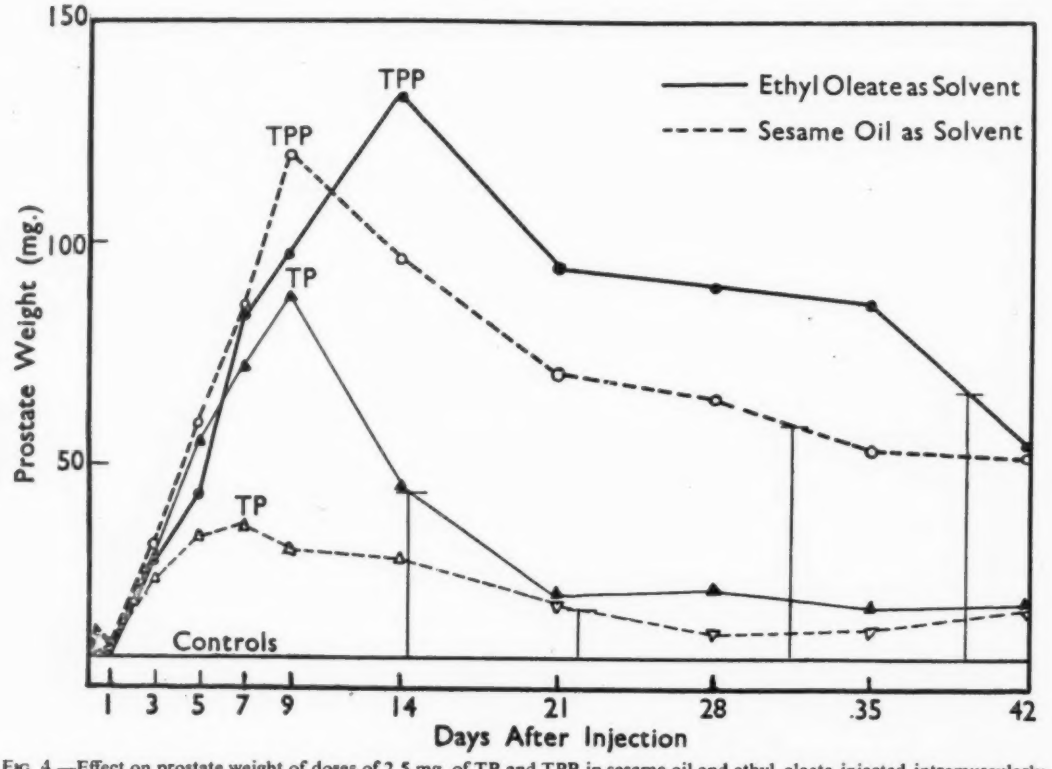


FIG. 4.—Effect on prostate weight of doses of 2.5 mg. of TP and TPP in sesame oil and ethyl oleate injected intramuscularly in castrated rats. Vertical lines as in Fig. 1.

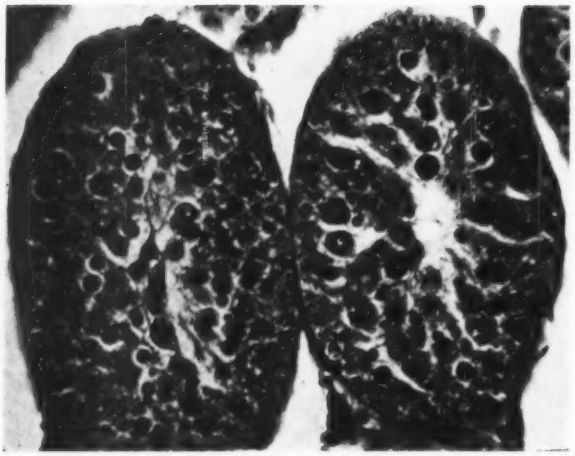


FIG. 5.

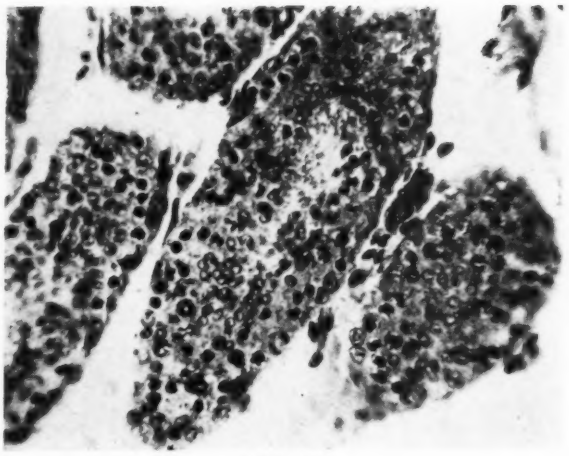


FIG. 6.

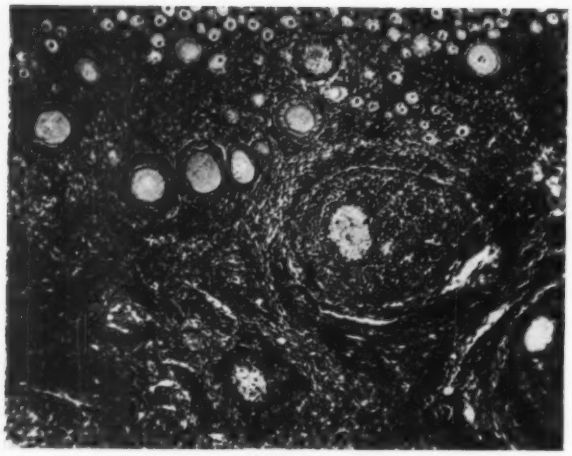


FIG. 7.

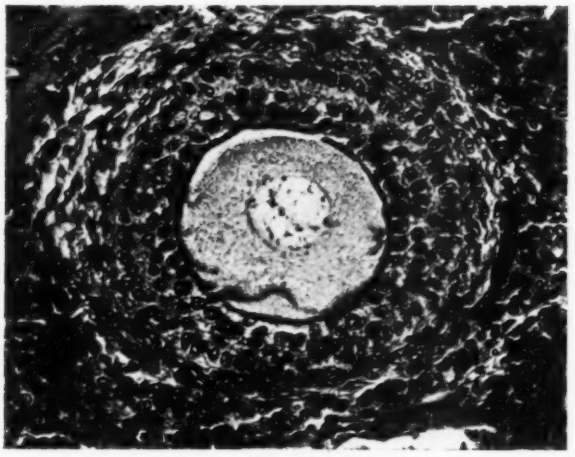


FIG. 8.

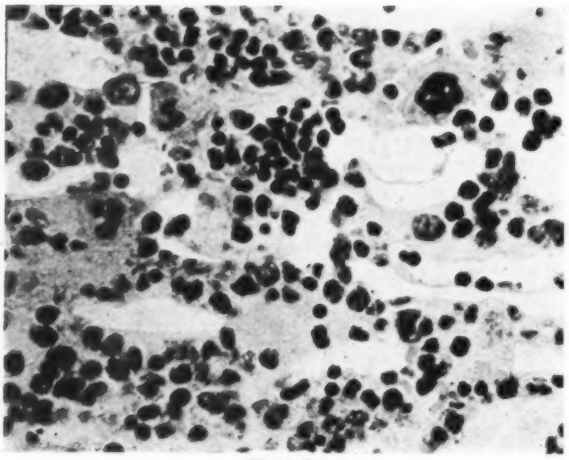


FIG. 9.

FIG. 5.—The seminiferous tubules of prepuberally castrated rat injected with 0.8 mg. testosterone phenyl propionate (× 275).

FIG. 6.—The seminiferous tubules of rabbit injected with 366 mg. testosterone phenyl propionate (× 220).

FIG. 7.—The ovary of rabbit injected with 366 mg. testosterone phenyl propionate (× 60).

FIG. 8.—High-power view (× 275) of the section shown in Fig. 7.

FIG. 9.—Red bone-marrow of rabbit injected with 366 mg. testosterone phenyl propionate (× 440).

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times as effective as the aqueous suspension as a vehicle for TPP when used subcutaneously in rats at a dose of 2.5 mg./0.1 ml.

The subcutaneous route was about 1.5 (seminal vesicles) and 1.3 (prostates) times as effective as the intramuscular route for TPP in ethyl oleate and about 2.8 (seminal vesicles) and 2.9 (prostates) times as effective as the intramuscular route for TPP in aqueous suspension in rats at a dose of 2.5 mg./0.1 ml.

Combining the most advantageous of the above results it was found that TPP in ethyl oleate given subcutaneously was about 7.9 (seminal vesicles) and 9.9 (prostates) times as potent as TP in sesame oil given intramuscularly to rats at a dose of 2.5 mg./0.1 ml.

Morphology of Testes.—It has been shown that androgens administered to immature and adult rats in low dosage may stimulate spermatogenesis, but that in larger doses they may cause atrophy of the testes and depression of spermatogenesis. TPP lowered testis weight and impaired spermatogenesis in a single dose of 0.8 mg.; this dose was found to affect meiosis, causing pyknotic degeneration of the nuclei of the primary spermatocytes, so that no later stages of spermatogenesis were observed (Fig. 5). The somatic, spermatogonial, Sertoli's, and Leydig cells were not affected. The peak of this activity was observed on the 7th day after injection, but by the 14th day definite recovery was noticed, suggesting that the depressant effect on spermatogenesis in the rat was only temporary.

Toxicity.—In rabbits treated with relatively large doses of TPP no pathological changes were observed on gross examination, except the reduction in size of the testes and the usual enlargement of seminal vesicles and prostate. The average total increase in body weight was 202 g. in rabbits treated with TPP and 228 g. in untreated control animals.

Microscopic examination of the various organs revealed significant changes only in the gonads and bone-marrow, these being observed in all rabbits treated with TPP.

A depressing effect on spermatogenesis was seen in the rabbit similar to that already reported in the rat with lower dosage, except that in the rabbit the pyknotic degeneration affected the nuclei of the secondary rather than the primary spermatocytes (Fig. 6) ; here again no further stages of spermatogenesis were seen, but in the lumina of the tubules there was some unidentified debris.

It is perhaps of interest that the ovarian response to excess TPP, in the two rabbits examined, was characterized by numbers of primary and growing follicles with the ova in premature stages of meiotic prophase (Figs. 7 and 8).

Investigation of the red bone-marrow of all the treated rabbits revealed no changes other than a stimulation of myeloid tissue with proliferation of eosinophil myelocytes, probably both the true and pseudo-eosinophil myelocytes (Fig. 9). There were no significant changes either in the splenic pulp or its cells, nor in nodules of lymphoid tissue, lymph nodes, or thymus.

There were no detectable pathological changes in other tissues examined (myocardium, lung, intestine, pancreas, liver, kidney) nor in other endocrine organs (pituitary, thyroid, parathyroid, islets of Langerhans, adrenal). Only in one of the female rabbits examined was there found a mild parenchymatous degeneration (cloudy swelling) of renal and hepatic epithelium ; this isolated finding may or may not be significant.

In rats injected with the emulsions there was a certain amount of local necrosis at the site of injection.

Discussion

It has been found that the complex androgens testosterone cyclopentylpropionate (TCP) and testosterone phenyl propionate (TPP) show greater potency and duration of effect than any testosterone compounds previously described. Up to the present, TCP and TPP have not been compared on rats, but a recent report from Overbeek (1952) shows that they have about the same potency in inducing capon comb-growth. It is clear from the present results that TPP has a higher potency and more prolonged action than testosterone propionate under the same conditions of administration.

An attempt at a 4-point test comparing TPP and TP gave a very similar result to that obtained from the activity-duration index adopted for this experiment.

It may be seen from the graphs that the weight of the target organs in these experiments increased at an almost identical rate for the first few days no matter what androgen, vehicle, or route was employed, and that the treatments which induced the greatest increase in weight were in general those which had the greatest duration of action.

The comparative failure of the forms designed for slow absorption—the aqueous suspensions and emulsions—must be offset against a number of favourable reports upon similar preparations ; it

may be that such forms fail unless a sufficiently large dose is given to deposit a layer of crystals deep enough to impede absorption (as occurs with subcutaneous implants); below this dose level the aqueous medium will naturally not hinder absorption. On the other hand, the low absorption rate of the hormone from an oil solution will probably be effective no matter how small the dose. It is noticeable from these results that the emulsions and suspensions compare particularly badly with the oil solutions when the dose is very low.

The effect of variations in the volume of injected material upon the target organs has not yet been tested, but it seems probable that the concentration of the dose will prove to be an important factor, especially with the crystal-depot type of preparation.

The advantage of ethyl oleate as a solvent was notable. Although its low viscosity makes it easy to inject, it appears not to result in rapid absorption; on the contrary, at the dosage of these experiments it would seem that the hormone is more slowly absorbed from this solvent than from any other medium tried.

Russell (1952) observed that ethyl oleate in rabbits caused local swelling, inflammation, and haemorrhage at the site of injection. In rats no significant local changes were observed by gross examination after subcutaneous or intramuscular injection of this solvent.

It is stated (Gaddum, 1952) that absorption from subcutaneous tissues is slower than from intramuscular ones, and the results clearly confirmed this, since both duration of effect and peak development were greater when the subcutaneous route was employed.

The involution of the target glands was interesting; as might be expected, the most stimulated organs tended to decrease more rapidly than less stimulated ones, as far as one can tell from the evidence of successive readings. More important, perhaps, is the observation that the seminal vesicles or prostates did not return to control size within six weeks of injection of a single moderate dose, the larger organs still being as much as 900% of the control weight.

The interference with spermatogenesis, which was clearly shown, is an indication of the efficacy of TPP as an inhibitor of anterior pituitary activity (Bottomley and Folley, 1938), since in rats this observation was made after a single dose of only 0.8 mg. This inhibition does not long outlast the administration of the hormone, however, and normal spermatogenesis was occurring again 14 days after the injection. The possible stimulating or maintaining effect of very small doses of TPP on the testis was not examined.

The effect of TPP on the ovary, although observed on only two rabbits, may prove to be of some importance in view of the part supposed to be played by ovarian testosterone in follicle ripening (Gaarenstroom and de Jongh, 1946), and has some parallel in the observation of Greep and Jones (1950) on rats.

Summary

1. 1,012 prepuberally castrated rats were used to test the potency of a new ester of testosterone, testosterone phenyl propionate (TPP), by comparison of its effects with those produced by testosterone propionate (TP) under similar conditions.

2. Preliminary tests on its potency, as judged by the stimulation of seminal vesicles and prostates at intervals after subcutaneous injection of small doses (0.2 and 0.8 mg.) in sesame oil, showed that TPP was about 2.7 times as potent an androgen as TP.

3. At a higher dose level of 2.5 mg. the potency of TPP as judged by a combined activity-duration index was at least four times that of TP when injected intramuscularly in ethyl oleate.

4. Ethyl oleate was found to be the most satisfactory vehicle for TPP, giving it greater and more prolonged effect than any other preparation tried; TPP was about one-and-a-half times as active in ethyl oleate as it was in sesame oil.

5. The subcutaneous route was found to give greater and more prolonged effect in rats than the intramuscular wherever these were compared; TPP in ethyl oleate was about one-and-a-half times as potent subcutaneously as it was intramuscularly.

6. TPP was found to be relatively free from toxic effects in rats and rabbits, but it will temporarily depress spermatogenesis even in moderate dosage.

7. Testosterone phenyl propionate appears to be a safe and potent long-acting androgen. It was especially effective in rats when injected subcutaneously in ethyl oleate.

We wish to thank Miss Margaret Harvie for her valuable assistance with the statistical work.

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OBSERVATIONS ON THE EFFECT OF TRIETHYLENE MELAMINE, AMINOPTERIN, AND A-METHOPTERIN ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

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In recent years numerous substances have been found capable of arresting the development of human leukaemia and certain solid tumours. In view of the practical importance of the avian leukosis complex (Darcel, Lancaster, and Gordon, 1952), it is surprising that the possible application of these drugs in its control has only received scant attention.

The purpose of the present paper is to present observations on the effect of aminopterin, A-methopterin, and triethylene melamine (T.E.M.) on the growth of transplantable lymphoid tumour strains isolated from cases of avian leukosis. Many workers have reported on the favourable effects of these drugs on the course of leukaemic and other neoplastic conditions in man and experimental animals. The first two are typical of the group known as folic acid antagonists, which it appears prevents the conversion of folic acid to the citrovorum factor, essential for nucleic acid synthesis (Nichol and Welch, 1951). It is possible that this interference with nucleic acid metabolism may partially explain the anti-leukaemic action of these drugs (Skipper, Bennett, and Law, 1952). Triethylene melamine is one of a series of tumour inhibitory agents developed by Rose, Hendry, and Walpole (1950). These resemble each other, and active nitrogen mustards, in that each molecule contains at least two alkylating groups. They produce chromosome fragmentation and bridge formation in proliferating cells. The cytotoxic action of this group might also partially be attributed to a specific inactivation of enzymes (Haddow, 1947).

The general characteristics of the tumour strains used in the present study, RPL 12, 16, and 19, have been described by Olson (1941), Burmester and Prickett (1945), and Burmester (1947). In experiments described by Darcel (1952), these three tumours killed 90–100% of chicks, inoculated subcutaneously, in 8–10 days. A tumour develops at the site of inoculation followed by rapid metastasis, in the absence of leukaemia, to the viscera, especially the liver.

METHODS

Crossbred (B.R. × Br. Leghorn) chicks were inoculated with tumour cell suspensions in the first few days of life. The chicks were maintained in small metal cloches and were fed *ad lib.* on a mash based on national standards.

The tumour cell suspensions were prepared in physiological saline with a special mincer (Craigie, 1949) from liver or pectoral muscle in which tumours were growing; they were filtered, before inoculation, through butter muslin and sintered glass filters (AG74 × 0) so that the tumour cells were more evenly distributed. The tumour suspension (0.25 ml.) was inoculated subcutaneously above the pectoral muscle. Concentrations of the tumour mince used corresponded to 1/10th of the original tumour in all experiments. Further dilutions (1/100, 1/1,000, and 1/10,000) were used in the studies on the effect of oral administration of T.E.M. and folic acid antagonists. The chicks were palpated daily for the calculation of mean time for first appearance of tumours in individual groups. In the experiments with aminopterin and A-methopterin the chicks were kept for three weeks only, but in the experiments with T.E.M. the observation period was extended to four weeks so that the mean survival times of the experimental groups could also be determined.

RESULTS

Triethylene Melamine (T.E.M.).—Rose, Hendry, and Walpole (1950) showed that T.E.M., given orally to rats, would inhibit tumour growth. This mode of administration in chicks is so convenient that it was used in three separate trials in which

the drug was given in the drinking water in concentrations up to 0.01% after inoculation. It had no significant effect on the length of time before tumours appeared or on the time that chicks survived after tumour transplantation. Although in this series of experiments no inhibitory effects of T.E.M. on the growth of these lymphoid tumours were demonstrated, there was evidence of a toxic effect in that chicks treated with the drug gained less weight. In the experiment in which 0.01% T.E.M. was administered in the drinking water, six of the twelve chicks not inoculated with tumour material died, the survivors weighing only 105.67± 14.9 g. in comparison with 151±5.25 g. for untreated. Corresponding mean weights for the surviving chicks which had been inoculated with tumour material were 89.38±5.58 g. and 143.67± 8.27 g. for the treated and untreated groups respectively. Chicks given T.E.M. at this dose level would have each received approximately 43, 96, and 160 mg. of the drug by the end of the second and third weeks respectively.

A different picture was seen when the drug was administered by the intraperitoneal route (Table I). In experiments 1 and 2 administration of the drug was delayed until palpable tumours appeared, whilst in experiments 3 and 4 administration was begun the day after implantation of tumour cells. In experiment 1 each chick received three daily intraperitoneal injections of a 0.001% solution of T.E.M. in distilled water. In experiment 2 four injections were given in six days and in experiments 3 and 4 five injections in 10 days.

TABLE I

THE EFFECT OF INTRAPERITONEAL ADMINISTRATION OF TRIETHYLENE MELAMINE ON THE GROWTH OF TRANSPLANTABLE AVIAN LYMPHOID TUMOURS

Exp. No.	Total Amount of T.E.M. Administered	Chicks Inoculated	Tumours		Deaths	
			Tumours Palpated	Mean Time for First Appearance in Days	No. of Deaths	Survival Time in Days
1	1·5 mg.	10	10	4·60±0·16	10	9·90±0·28
	0·75 „	10	10	4·80±0·13	10	12·00±0·75
	0	9	9	4·67±0·15	9	7·44±0·18
2	0·25 „	10	10	9·10±0·10	7	13·57±0·43
	0	10	10	9·20±0·13	9	12·22±0·33
3	0·31 „	10	9	8·56±0·33	8*	12·50±0·57
	0	10	10	6·10±0·10	10	9·30±0·15
4	0·31 „	10	10	5·40±0·34	10†	12·10±0·85
	0	10	10	4·10±0·10	10	6·90±0·18

* Two were killed with tumours 17 days after inoculation. One showed a very large ulcerated pectoral tumour. In both cases there was tumour involvement of the proventriculus.

† One died at 10 days from causes apparently not associated with tumour growth.

In experiments 1 and 2 tumour strain RPL 19 was used, and in experiments 3 and 4 RPL 12.

Other chicks were treated with T.E.M. in the same way, but were not implanted with tumour cells. Those which received 1.5 mg. died after 9.90±0.18 days and those which were given 0.75 mg. died after 12.00±0.78 days. Of the others, which received 0.31 or 0.25 mg., only two out of thirty died within the experimental period. It will be seen from Table I that in spite of these toxic effects of T.E.M. the chicks implanted with tumours and given T.E.M. lived longer than untreated chicks. In experiment 1 the untreated controls survived only 7.44 days, those which received 0.75 mg. 12.00 days ; 1.5 mg. T.E.M. reduced the survival time to 9.90 days because of its own toxic effects. In experiment 2 the dose of T.E.M. was less and tumour implanted chicks survived only a day longer than the untreated controls. In experiments 3 and 4, in which the drug was given before tumours appeared, there was a significant increase both in the time which elapsed before tumours appeared and in the survival time.

Aminopterin and A-methopterin.—In preliminary experiments chicks which were given five daily doses of aminopterin and A-methopterin in aqueous suspension (0.1 and 1.0 mg. of each drug per day), administered directly into the crop, showed a significant gain in weight compared with untreated controls. Only one out of 29 chicks died in this series.

The effect of the administration of aminopterin and A-methopterin on the growth of RPL 12 was tested by implanting groups of ten chicks with concentrations of tumour suspensions equivalent to 10^{-1}–10^{-4} of the original undiluted tumour mince. Five chicks in each group were given 0.01 mg. of the drug daily. The times during which tumours developed varied from 6–7 days from the chicks receiving the 10^{-1} suspension to 10–11 days for chicks receiving the 10^{-4} suspension. No significant differences were observed between the treated and untreated groups in the time for appearance of tumours.

DISCUSSION

In T.E.M. treated chicks in which the drug was given parenterally before the appearance of tumours, the latter took longer to become apparent, as judged by palpation, and there was an increased survival time. In the detection of tumours there is naturally a subjective error, but this is surprisingly small for these rapidly growing tumours (Darcel, 1952). The absence of any significant effect on tumour growth when the drug was given by the oral route is difficult to explain. The marked reduction in growth rate following treatment, both in tumour inoculated and uninoculated

chicks, suggests that absorption of the drug had occurred. This drug is not particularly stable, and it is possible that through the presence of reactive contaminants in the drinking water and intestine it might have been altered to an inactive but still toxic form.

No increase in the time taken for tumours to appear in treated chicks was observed in the experiments with aminopterin and A-methopterin. However, since there was no evidence of toxic effects when these drugs were given orally, and in view of the finding by Little *et al.* (1945) that a few doses of 10 micrograms given intraperitoneally to chicks were highly toxic, it must be assumed either that there is very little absorption in the intestinal tract or that an alteration of the drugs takes place. The fact that a significant weight increase occurred in chicks receiving aminopterin suggests that it is the latter explanation that is correct. Under these circumstances it is not surprising that aminopterin and A-methopterin given orally failed to influence tumour growth. In this connection it is of interest that the growth of the Rous sarcoma is unaffected by the administration of aminopterin in the food (Ringsted, 1952), but inhibited when this drug is given parenterally (Little *et al.*, 1945).

The absence of an effect of T.E.M. on the survival time when the drug was given parenterally after palpable tumours appeared recalls the experiments of Geisse and Kirschbaum (1950) with urethane and sodium arsenite on the course of mouse leukaemia. The maximum effect was obtained only if these were given shortly after transplantation of the leukaemia.

The inhibitory effect of parenteral administration of T.E.M. on the growth of these lymphoid tumours suggests that this compound might be given field trials in attempts to prevent the development of spontaneous lymphomatosis. Such attempts should be postponed until more is known about the toxicity and possible carcinogenic activity of this compound.

Summary

1. Triethylene melamine given orally had no significant effect on the growth of transplantable avian lymphoid tumours. Given parenterally before the appearance of palpable tumours, the drug had some inhibitory effect on tumour growth but was highly toxic. If treatment was delayed until the tumour appeared there was no significant effect on the survival time of inoculated chicks.

2. Aminopterin and A-methopterin given orally to chicks bearing tumour strain RPL12 had no significant effect on growth. The absence of toxic effects following much heavier dosage suggests that the chicks did not absorb the drug in sufficient quantity.

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A NOMOGRAM FOR ASSAYS IN RANDOMIZED BLOCKS

BY

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This nomogram results from an extension of the idea presented by Healy (1949) in which the fiducial or confidence limits of an assay were found at the intersections on the branches of a hyperbola of a straight line whose position was determined by the mean potency, slope, and residual error of the assay, the error being estimated from the mean of the ranges within the sets of responses to each dose. The same principle has been extended to cover assays arranged in randomized blocks with the particular case of tuberculin assay in mind (*British Pharmacopoeia*, 1948); some modifications enable a single graph to be applied to assays of different sizes. Error is again estimated from range, and the method presented fulfils the condition (Finney, 1952a) that the deviations for the interactions with blocks of "slope" and "materials" should be eliminated from the error variance estimate. Identical computations on a series of 12 similar assays are presented to show the practical implications of Finney's argument and to test the value of the range in estimating the error of these assays.

The nomogram illustrated (Fig. 1) is constructed for the particular case of the 6-point assay in randomized blocks with a dose ratio of 4:1; this arrangement is commonly used in the assay of tuberculin. The modifications required for other designs are specified in a later section.

Numerical Example

The data in this example (Table I) are part of the results of an assay of two tuberculins, being the diameters in mm. of red weals measured 24 hours after the intradermal injection of tuberculin into sensitized guinea-pigs. The responses to the two materials are entered in columns *a*, *b*, *c* and *g*, *h*, *i* respectively; the other nine columns are filled in with the results of simple operations on these six, the operations being specified at the head of each column. The same operations on the totals of columns *a*, *b*, *c* and *g*, *h*, *i* will check with the algebraic sum of each of the other nine columns.

The statistics required for using the nomogram are *D*, *B*, and *R*, the derivation of each being illustrated in the example. The values of *P*, C_1, C_2 should also be estimated and used to check the validity of the assay. For a valid assay, *P* should not be greater than $u'R$; C_1 and C_2 should not exceed $\sqrt{3u'R}$ (u', for different sizes of assay, has

TABLE I

SPECIMEN COMPUTING SHEET

	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)	(*i*)	(*j*)	(*k*)	(*l*)	(*m*)	(*n*)	(*o*)
Block No.	Responses to Standard			$a+b+c$	$c-a$	$a+c-2b$	Responses to Unknown			$g+h+i$	$i-g$	$g+i-2h$	$(k-e)$	$(f+l)$	$(f-l)$
	$\frac{1}{1600}$	$\frac{1}{400}$	$\frac{1}{100}$				$\frac{1}{1600}$	$\frac{1}{400}$	$\frac{1}{100}$						
1	8	13	16	37	8	−2	4	8	14	26	10	2	2	0	−4
2	12	15	15	42	3	−3	9	12	16	37	7	1	4	−2	−4
3	15	16	19	50	4	2	12	15	20	47	8	2	4	4	0
4	8	14	17	39	9	−3	10	10	16	36	6	6	−3	3	−9
5	14	14	17	45	3	3	10	14	18	42	8	0	5	3	3
6	9	16	17	42	8	−6	12	12	16	40	4	4	−4	−2	−10
7	9	14	18	41	9	−1	8	14	16	38	8	−4	−1	−5	3
8	6	12	18	36	12	0	4	10	19	33	15	3	3	3	−3
Total	81	114	137	332	56	−10	69	95	135	299	66	14	10	4	−24
												Ranges	9	9	13

From column totals, compute: $D=j-d=-33$. $B=e+k=122$. $M=4D/3B=-0.361$.
From ranges: $R=\sqrt{3m+n+o}=37.6$. $R/B=0.308$.
For validity tests, compute: $P=e-k=-10$. $C_1=f+l=4$. $C_2=f-l=-24$. $Ru'=14.9$. $\sqrt{3Ru'}=25.8$.
Standard deviation (if required) $=R/(6\sqrt{3}.d_N)$.

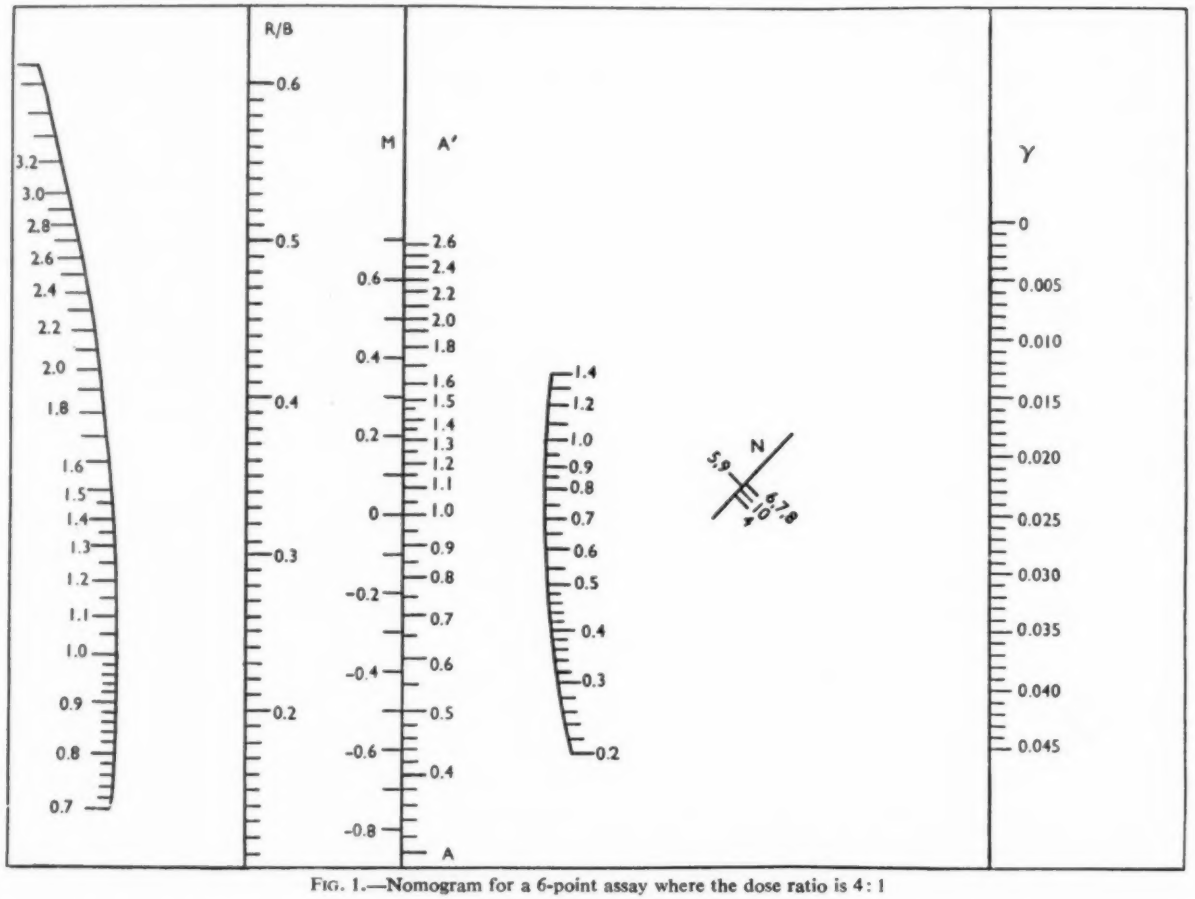

FIG. 1.—Nomogram for a 6-point assay where the dose ratio is 4 : 1

been tabulated (Table II)). If the validity of the assay is not in doubt, calculation of the ratios $4D/3B$ and R/B completes the computations.

TABLE II

TABLE OF u' (5% SIGNIFICANCE LEVEL)

No. of Blocks	6-point Assay	8-point Assay
4	0·415	0·410
5	0·398	0·398
6	0·392	0·396
7	0·393	0·397
8	0·396	0·401
9	0·400	0·408
10	0·405	0·413

Construction of the Nomogram

The nomogram can conveniently be drawn on a paper whose sides are approximately in the proportions 4 : 3. The horizontal scale (x) is graduated from -20 to $+30$ and the vertical scale (y) is -0.9 to $+1.1$. In the example from which the illustration was prepared (Fig. 1) the unit of x was 0.5 cm. and the unit of y 10 cm. Vertical axes are drawn at $x=0$ and $x=30$. On the former a scale of μ is graduated, covering the range -0.9 to $+0.7$, the units of μ being equal to the units of y. (μ is the quantity estimated by $M=4D/3B$). The other scale (AA') on the axis is one of 4^{μ}, i.e., antilog (μ log 4), which gives the relative potency corresponding to the value of μ graduated. The co-ordinates set out in Table III will enable the curves shown in Fig. 1 to be drawn without further calculation. The values of relative potency are those to be marked when the dose ratio is 4 : 1. The finer calibrations may be interpolated by eye; if greater precision is desired, there is an easy graphical method (Allcock and Jones, 1950) that obviates further computation. The position of the curves is not affected by alterations in the dose ratio or in the size of the assay. It will be appreciated that it is only necessary to draw the left-hand branch between the values 0.7 and 4.0 and the right hand branch between the values 0.2 and 1.4. For other values of the dose ratio, the curves may be calibrated quite rapidly once the scale AA' has been drawn. Where a line drawn through the point (30, 0.75) and any point on $x=0$ cuts

TABLE III
CO-ORDINATES FOR THE CURVES IN FIG. 1

Relative Potency	Left-hand Curve		Relative Potency	Right-hand Curve	
	x	y		x	y
0·70	−14·82	−0·755	0·20	8·58	−0·614
0·80	−14·65	−0·606	0·22	8·46	−0·573
0·90	−14·57	−0·477	0·24	8·36	−0·534
1·00	−14·55	−0·364	0·26	8·26	−0·498
1·10	−14·57	−0·262	0·28	8·18	−0·463
1·20	−14·62	−0·170	0·30	8·10	−0·431
1·30	−14·70	−0·085	0·32	8·03	−0·401
1·40	−14·79	−0·007	0·34	7·97	−0·372
1·50	−14·90	0·065	0·36	7·91	−0·345
1·60	−15·02	0·133	0·38	7·86	−0·318
1·70	−15·14	0·197	0·40	7·82	−0·293
1·80	−15·28	0·258	0·50	7·64	−0·182
1·90	−15·42	0·315	0·60	7·52	−0·088
2·00	−15·56	0·370	0·70	7·45	−0·007
2·20	−15·86	0·473	0·80	7·41	0·064
2·40	−16·17	0·568	0·90	7·39	0·128
2·60	−16·48	0·656	1·00	7·39	0·185
2·80	−16·79	0·739	1·10	7·39	0·237
3·00	−17·10	0·817	1·20	7·40	0·284
3·20	−17·41	0·891	1·30	7·42	0·328
3·40	−17·72	0·961	1·40	7·45	0·369
3·60	−18·02	1·029			
3·80	−18·32	1·093			
4·00	−18·62	1·155			

the curves, the 3 intersections will bear the same calibration.

A vertical scale on the line $x=-8$ is marked with a suitable range of values of R/B. A scale of γ is marked on the axis $x=30$ from the equation

$$\gamma=(0.75-y)/30$$

On the line joining $R/B=0$ to $\gamma=0$ a short scale of N may be graduated, the calibrations for integral values of N being found at the intersections of lines joining the point $R/B=0.6$ to the points at which γ is numerically equal to $u'/10$ (Table II): the scale of γ is only required for calibrating the scale of N and may then be erased.

Using the Nomogram.—A straight-edge is placed across the appropriate points on the scales of R/B and N (0.308 and 8 respectively in the example) and the intersection of this line on the γ scale marked. The fiducial limits may then be read off at the intersections on the curves of the line joining this point to the observed value of $M(-0.361)$. The relative potency corresponding to the observed M can be read off without further computation from the scale AA'.

THEORY

The fiducial or confidence limits for a ratio derived from a 6-point assay may be expressed as

$$(4D-3B\mu)^2=Nt^2s^2(96+36\mu^2) \quad \ldots\ldots(1)$$

where $\mu=\log$ (potency ratio)/log (dose ratio) and D and B are explained in Table I; s^2 is the error variance of a single observation and t the appropriate deviate of the Student distribution. Since a range estimate of s is used (Table I) in place of the least squares estimate, the statistic u (Lord, 1947) must be substituted for Student's t. Taking m, n, and o (Table I) as the ranges appropriate to the estimation of s, we may write $s=R/(d_N.6\sqrt{3})$, where $R=m\sqrt{3}+n+o$ and d_N is the expected value of the range in samples of N randomly selected from a normal population with unit standard deviation; this quantity has been tabulated by various writers, the table (Table 5.5) given by Snedecor (1946) being probably the most accessible. If we now write $\gamma=\{R/B\}\ \{u\sqrt{N}/18\sqrt{3}d_N\}$, equation (1) may be written

$$(M-\mu)/\gamma=\pm\sqrt{(96+36\mu^2)} \quad \ldots\ldots(2)$$

This is equivalent to

$$\begin{vmatrix} x_1 & y_1 & 1 \\ \lambda & -\gamma\lambda & 1 \\ 0 & M & 1 \end{vmatrix}=0 \quad \ldots\ldots(3)$$

where λ is an arbitrary constant and

$$x_1=\frac{\pm\lambda\sqrt{(96+36\mu^2)}}{\lambda\pm\sqrt{(96+36\mu^2)}}, \qquad y_1=\frac{\lambda\mu}{\lambda\pm\sqrt{(96+36\mu^2)}}$$

The form of equation (3) is such that the points with co-ordinates (x_1, y_1), $(\lambda, -\gamma\lambda)$, and $(0, M)$, say F, G, and H, are collinear. Owing to the introduction of the constant λ, the locus of G provides a scale of γ at finite distance. The corresponding property of Healy's nomogram is, in our notation, that the points F' and H' with co-ordinates $(\pm\sqrt{(96+36\mu^2)}, \mu)$ and $(0, M)$ lie on a line of gradient $-\gamma$; that is, they are collinear with the point G' given by $x'=\infty$, $y'/x'=-\gamma$. The two schemes are connected by the projective transformation

$$x=\frac{\lambda x'}{\lambda+x'}, \qquad y=\frac{\lambda y'}{\lambda+x'},$$

which converts F', G', H' into F, G, H respectively.

Since only positive values of γ are relevant, it is convenient to shear the right-hand side of the nomogram (including the scale of γ) vertically upwards. This may be effected by adding λ' times all x-co-ordinates to the y-co-ordinates, so that the determinant becomes

$$\begin{vmatrix} \pm\lambda\sqrt{(96+36\mu^2)} & \lambda\{\mu\pm\lambda'\sqrt{(96+36\mu^2)}\} & 1 \\ \lambda\pm\sqrt{(96+36\mu^2)} & \lambda\pm\sqrt{(96+36\mu^2)} & \\ \lambda & \lambda(\lambda'-\gamma) & 1 \\ 0 & M & 1 \end{vmatrix} \quad \ldots\ldots(4)$$

Values of $\lambda=30$ and $\lambda'=0.025$ were found to be convenient; the determinant, with the values substituted leads at once to a nomogram with scales given by the three sets of co-ordinates

(i) $\left(\frac{\pm 30\sqrt{(96+36\mu^2)}}{30\pm\sqrt{(96+36\mu^2)}}, \frac{30\mu\pm 0.75\sqrt{(96+36\mu^2)}}{30\pm\sqrt{(96+36\mu^2)}}\right)$

(ii) $(30,\ 0.75-30\gamma)$

(iii) $(0,\ M)$

The scale of N (the number of blocks) is derived from the scales of γ and R/B from the relationship $\gamma(B/R)=(u\sqrt{N})/(18\sqrt{3}d_N)$; here the right-hand side is equal to $u'/6$ in the notation of Table II. Any convenient position and graduation of a vertical scale R/B may be used, provided only that it is graduated in the opposite sense to the scale of γ.

The scale AA' of potency ratios (Fig. 1) is a scale of 4^{μ}, since $\mu=\log$ (potency ratio)/log 4. If the dose ratio is reduced, the scale expands and the nomogram will have to be compressed. Conversely, a wider dose ratio calls for an expansion of the nomogram. These alterations are effected by changing the unit of y. It will usually then be an advantage to alter the degree of shear, increasing λ' when the dose ratio is less than 4:1; alterations of λ' are less necessary if the dose ratio is increased.

Modifications for the 8-point Assay

The estimate of μ is now $M=\frac{5D}{B}$, where D and B are the totals corresponding to the contrasts which Finney (1952b, p. 155) symbolizes as L_p and L_1. The co-ordinates of the hyperbola become

$$\left(\frac{\pm\lambda\sqrt{(5+\mu^2)}}{\lambda\pm\sqrt{(5+\mu^2)}}, \frac{\lambda\{\mu\pm\lambda'\sqrt{(5+\mu^2)}\}}{\lambda\pm\sqrt{(5+\mu^2)}}\right)$$

Suitable values of λ and λ' will have to be chosen for the particular conditions of assay. The value of R can be estimated from 5 ranges, each of which is divided by the square root of the sum of squares of the coefficients (Finney, 1952b) used to evaluate the interaction concerned. If m, n, o, p, and q are respectively the ranges within the contrasts that Finney (1952b, p. 155) symbolizes as L_1', L_2, L_2', L_3, and L_3' and we write

$$R=\sqrt{5}(n+o)+m+p+q,$$

then $s=R/(10\sqrt{10}d_N)$, from which it follows that $u'=(u\sqrt{N})/5d_N$ is the appropriate factor for testing the significance of the analogue of P (Table I) and $u'/\sqrt{5}$ is the factor for testing the analogues for C_1 and C_2. (In Finney's notation, P, C_1, and C_2 are respectively L_1', L_2, and L_2'.) A range of values of u' is included in Table II. It should be noted that here we put

$$\gamma(B/R)=(u\sqrt{N})/5d_N=u',$$

so that the values of u' also provide the basis for calibrating the scale of N.

Relative Value of Range and Root-mean-square in Evaluation of Fiducial Limits

Both range and root-mean-square calculations have been applied to the data of 12 assays in which the potencies of the same two tuberculins were compared on sets of 8 guinea-pigs. One set of standard deviations was estimated from range by the method demonstrated in Table I; a second set was derived from least squares, using the same set of degrees of freedom (the error term in Table IV).

TABLE IV

SCHEME FOR THE ANALYSIS OF VARIANCE OF A 6-POINT ASSAY IN 8 RANDOMIZED BLOCKS

	d.f.
Treatments	5
Blocks	7
Materials × blocks	7
Slope × blocks	7
Error	21
Total	47

Five per cent. fiducial limits were calculated from the two sets of standard deviations, using the value of $u(2.07)$ for the range estimates and $t(2.08)$ for the least-squares estimates. The fiducial limits were then expressed as percentages of the mean. It will be seen from Table V that the intervals between the upper and lower percentage limits based on range compare satisfactorily with the corresponding intervals based on the root-mean-square estimate. (The mean interval on the scale of a normal variate is of course $2\times 2.07=4.14$ in units of the standard deviation, when the range estimate is used. For the root-mean-square estimate, since the expected value of $\sqrt{(\chi^2/21)}$ with 21 degrees of

TABLE V

(a) INTERVALS BETWEEN UPPER AND LOWER FIDUCIAL LIMITS WHEN THE LIMITS HAVE BEEN EXPRESSED AS PERCENTAGES OF THE MEAN RELATIVE POTENCY. (b) STANDARD DEVIATIONS

	(a)		(b)	
Assay No.	Range Estimate	Least Squares Estimate	Range Estimate	Least Squares Estimate
1	78	79	1·337	1·357
2	83	84	1·396	1·402
3	84	85	1·322	1·346
4	73	72	1·453	1·439
5	66	62	1·414	1·326
6	55	64	1·236	1·398
7	68	70	1·009	1·028
8	85	82	1·658	1·610
9	87	80	1·492	1·381
10	55	60	1·036	1·129
11	69	70	1·163	1·167
12	95	92	1·743	1·685
Mean ..	74·83	75	1·3541	1·3557

freedom is found to be 0.9882, the corresponding figure is $2 \times 2.08 \times 0.9882 = 4.11$. Thus the observed results are slightly more favourable to the range estimate than can be expected in the long run.)

The analysis of variance (Table IV) is constructed on the principle recently demonstrated by Finney (1952a), that the interactions of " materials " and of " slope " with blocks should be removed from the error sum of squares, because each contains a component that is a function of the block-to-block variation in the slope of the response line, and does not affect the variability of the relative potency estimate.

Bliss (1952) has presented some data in which these interactions were negligible, and surmises that the theoretical argument may have little practical importance; an investigation of the above 12 tuberculin assays revealed an entirely different situation, for in these assays the interactions were often of considerable magnitude; in consequence the errors of the assays were often grossly over-estimated by the earlier procedure. It is also noteworthy that the correct estimate is much more stable from one assay to another than the estimate commonly used which, in our example, would be based on 35 d.f. The separate mean squares with 14 and 21 d.f. are given in Table VI; Bartlett's test for homogeneity gives $\chi^2_{(11)} = 8.60$ for the error, but 32.15 for the pooled interactions.

TABLE VI

ERROR MEAN SQUARE (21 D.F.) AND POOLED INTERACTION MEAN SQUARE (14 D.F.) FROM TWELVE ASSAYS OF THE SAME TUBERCULINS

Assay No.	Error Mean Square	Mean Square for Interactions, of Slope and Materials (with Blocks)
1	1·843	3·843
2	1·966	0·724
3	1·811	0·942
4	2·071	2·167
5	1·758	4·130
6	1·954	4·549
7	1·057	2·563
8	2·593	7·353
9	1·906	1·581
10	1·274	4·975
11	1·363	2·206
12	2·839	4·657
Mean ..	1·8696	3·3075

SUMMARY

1. A scheme, based on a nomogram, has been devised for the routine computation of the 5% fiducial limits of the relative potency of an assay arranged in randomized blocks. An illustration is given of the appropriate method of obtaining a range estimate of error.

2. The theory and construction of the nomogram are discussed; a table of the co-ordinates of the curves is provided to facilitate the construction.

3. Range is shown to be a reliable estimator of the error of tuberculin assays.

4. The magnitude of the interactions of " materials " and " slope " with blocks has been investigated. It is shown that in tuberculin assays they may be large and must therefore be eliminated from the estimate of error.

The data on tuberculin assays were kindly placed at our disposal by Mr. A. B. Paterson, of the Veterinary Laboratory, Weybridge. We wish also to acknowledge helpful discussions with Mr. M. J. R. Healy.

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SYNTHETIC AMOEBICIDES: PART II. THE ANTI-AMOEBIC ACTION OF QUINOXALINE-1:4-DIOXIDE AND SOME DERIVATIVES

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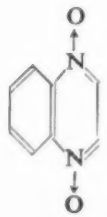
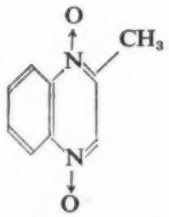
McIlwain (1943) prepared quinoxaline-1:4-dioxide and homologues as possible antagonists of vitamin K. He showed that these compounds possessed antibacterial properties against *Streptococcus haemolyticus* and *Corynebacterium diphtheriae*; further antibacterial studies with compounds of this class were made by Wiedling (1945), Frisk (1946), and Iland (1948).

In the course of routine screening tests of compounds as possible amoebicides, we selected (*a*) compounds which might function as antagonists of vitamins, and (*b*) those which might interfere with the oxidation-reduction potential of the parasites' environment. Whereas we had no evidence that *Entamoeba histolytica* was vitally dependent upon vitamin K, the work of Chang (1946) and Bradin and Hansen (1950) indicated the sensitivity of this parasite to changes in oxidation-reduction potential of the medium in which it was cultivated. Among the compounds examined was 2-methylquinoxaline-1:4-dioxide (ref. No. 7331), which was found to be highly active against an experimental amoebic infection of rats. Other quinoxaline dioxides behaved similarly.



Quinoxaline-1:4-dioxide (ref. No. 8173)



2-Methylquinoxaline-1:4-dioxide (ref. No. 7331)

We have found, however, that the quinoxaline oxides do not behave as oxidizing agents towards typical oxidation-reduction indicators, and that their biological activity is not antagonized by 1:4-naphthoquinone or vitamin K. Their mode of action, therefore, remains unexplained.

METHODS AND MATERIALS

Relative Therapeutic Potency.—The experimental amoebic infection of young rats (Jones, 1946) was used to assay the anti-amoebic effect of the compounds tested. Young rats (weighing 20–35 g.) were anaesthetized with ether and then injected intracaecally with 0.2 ml. of a suspension of *E. histolytica* harvested from several two-day-old cultures. In the morning and afternoon of each of the two days after this operation, a suspension or solution of the compound was administered orally. On the fifth day after the operation, the rats were killed and a careful assessment was made of the average degree of amoebic infection present in the caecum. By comparison of this figure with that obtained from a group of untreated controls a measure of the therapeutic effect could be made (Jones, 1946). Each of the compounds of this series was examined repeatedly, and that dose which would consistently produce a significant therapeutic effect was determined. This dose was used to characterize the relative potencies of the compounds.

Materials.—Quinoxaline-1:4-dioxide (8173) is a yellow crystalline substance, m.p. (when heated rapidly) 241–243° C. (decomp.). It is soluble in cold water to the extent of about 1.5%. Solutions of the drug are relatively unstable to visible or ultra-violet light, and in all the investigations described below precautions were taken to prevent photochemical-decomposition.

2:3-Dimethylquinoxaline-1:4-dioxide (7218) is a pale yellow crystalline substance, m.p. 193–194° C. It is soluble in cold water to the extent of 10%. In solution the substance is somewhat photo-labile, and, as with 8173, precautions were taken to prevent photochemical-decomposition. 7218 could also be estimated spectrophotometrically in blood or plasma.

6-Chloro-2:3-dimethylquinoxaline-1 : 4-dioxide (8218) is a pale yellow crystalline substance, m.p.

175–176° C. It is only sparingly soluble in water. As with 8173 and 7218, suspensions of the drug in water are unstable to light, and precautions were taken to prevent photochemical decomposition. 8218 was more slowly and less completely absorbed than 8173, as determined by spectrophotometrical studies of blood from mice dosed with the drug.

RESULTS

From the results shown in Table I it was apparent that quinoxaline-1 : 4-dioxide (8173), its lower homologues, and the related 8218 (6-chloro-2 : 3-dimethylquinoxaline-1 : 4-dioxide) were the most highly active of the series. With all of these compounds *E. histolytica* persisted in a proportion of the treated rats after the lesions were healed. For example, 8173, although having a very striking effect on the lesions at 2.5 mg./kg., eradicated *E. histolytica* from only 56% of the rats. Emetine hydrochloride produced a comparable effect at 0.6 mg./kg.

TABLE I
RELATIVE THERAPEUTIC POTENCY OF MEMBERS OF A SERIES OF QUINOXALINE-1:4-DIOXIDES (AGAINST EXPERIMENTAL AMOEBIASIS OF RATS)

Code Number	Substituents	Minimal Consistently Effective Therapeutic Dose
		mg./kg. b.i.d.×2
8173	None	2·5
7331	2-methyl	5
7218	2 : 3-dimethyl	5
9804	2-methyl-3-ethyl	1
10,323	2-methyl-3-*n*-propyl	5
10,376	2-methyl-3-*iso*propyl	10
9630	2-methyl-3-*n*-amyl	10
11,315	2-methyl-3-phenyl	25
8218	6-chloro-2 : 3-dimethyl	5
10,856	2-ethyl	5
9915	2 : 3-diethyl	1
10,931	2-ethyl-3-*n*-propyl	10
9833	2 : 3-di-*n*-propyl	25
8175	2 : 3-diphenyl	10

To determine, roughly, the relative oral toxicities of the more active compounds, groups of six male albino mice of uniform weight (18–20 g.) were dosed orally twice daily (once daily on the sixth and seventh day) for 12 days. The LD50 for each compound was estimated and the results are given in Table II.

It was necessary, at an early stage in these investigations, to select members of this series of compounds for further therapeutic and toxicological investigation, with a view to their subsequent trial in man. From the evidence of their therapeutic action in rats, their toxicity in mice, and their mode of absorption and excretion, 8173 (unsubstituted), 7218 (2 : 3-dimethyl-), and 8218 (6-chloro-2 : 3-dimethylquinoxaline-1 : 4-dioxide) were selected for further study. After oral dosage, 8173 was found to be well absorbed into the blood stream, and rapidly excreted; 7218 was rather more slowly absorbed, but was excreted much more slowly; 8218 was rather poorly absorbed. The minimal concentrations of these three substances causing inhibition of growth of *E. histolytica* in a simple liquid medium (Jones, 1946) were respectively 1 : 640,000, 1 : 80,000, and 1 : 640,000. The inhibitory concentration of emetine hydrochloride under these conditions was 1 : 3,000,000.

TABLE II
RELATIVE CHRONIC ORAL TOXICITIES, IN MICE, OF QUINOXALINE-1 : 4-DIOXIDE AND SEVERAL RELATED COMPOUNDS
Dosing: twice daily (once daily on the 6th and 7th days) for 12 days

Code No.	Substituents	Estimated LD50 in mg./kg.	95% Limits (mg./kg.)
8173	None	135	70–250
7331	2-methyl	80	65–100
7218	2 : 3-dimethyl	360	155–850
9804	2-methyl-3-ethyl	130	115–150
9630	2-methyl-3-*n*-amyl	1,085	745–1,575
9915	2 : 3-diethyl	175	150–195
8218	6-chloro-2 : 3-dimethyl	500	425–575

8173 (Quinoxaline-1 : 4-dioxide)

When administered orally to rats or monkeys in doses greater than 10 mg./kg., 8173 could be estimated spectrophotometrically in the blood or plasma, and was apparently present in unaltered form. Maximal blood concentrations were obtained 1–2 hours after dosing. Thereafter, a small proportion of the drug was excreted, but the greater part was rapidly metabolized. There was apparently no accumulation of drug in the blood of rats even after the daily oral administration of 100 mg./kg. for three weeks. The compound was retained to some extent in the metabolically inert tissues (fat), but was rapidly destroyed in the more active tissues such as liver and kidney. Mice, rats, and rabbits survived repeated daily oral doses of 100 mg./kg. up to three weeks; the LD50 in rats was 150 mg./kg. under these circumstances. In one monkey, daily oral doses of 250 mg./kg. caused slight anaemia and granulocytopenia; three daily oral doses of 500 mg./kg. caused the death of one monkey, which at necropsy showed fatty degeneration of the liver and tubular damage in the kidney; 100 mg./kg. did not affect the blood picture in the dog. No action was observed on blood pressure, respiration, or plain muscle. The toxic action of the drug when given to rats in doses exceeding 100 mg./kg. was directed mainly against the kidney, which showed glomerular haemorrhages and tubular desquamation, and the liver, which suffered fatty degeneration.

Daily oral doses of 250 mg./kg. given to one monkey caused cysts of *E. histolytica* to disappear from the stools ; five daily oral doses of 50 mg./kg. also caused the parasites to disappear, but they reappeared four weeks afterwards. Five daily oral doses of 20 mg./kg. had no therapeutic effect.

One kitten and one puppy were cured of experimentally induced amoebic dysentery by the administration of four daily doses of 5 mg./kg. and 20 mg./kg. of drug respectively.

Clinical Trial in Man.—Quinoxaline-1 : 4-dioxide (8173) was tried in 14 patients suffering from amoebiasis. Dr. A. R. D. Adams, of the Liverpool School of Tropical Medicine, carried out the trial ; the results can be summarized as follows.

Eight patients presenting minimal clinical symptoms, and consistently passing cysts of *E. histolytica* in the faeces, received 8.5 to 19 mg./kg./day in divided daily doses (3–5 for 21 days). Two of the eight ceased to pass cysts on the fourth and ninth days respectively of a 21-day course of thrice-daily treatment with 200 mg. (8.5–9.5 mg./kg.). The remaining six patients continued to pass cysts as before treatment.

Four patients suffering from acute amoebic dysentery were treated with 5.3–20 mg./kg. of the drug daily for 7–22 days. Two patients (Indian) responded well ; in one the acute attack had subsided by the third day and the parasites vanished on the fifth day. These two patients had 150 and 200 mg. 4-hourly five times daily for 17 and 21 days respectively (16.7 and 20 mg./kg.). The other two patients (both Europeans) showed no symptomatic response and parasites remained present. In one of these patients, treatment was abandoned after seven days because of toxic effects.

Two patients suffering from acute amoebic hepatitis responded to treatment within 2–3 days. Symptoms subsided and white cell counts diminished. Treatment was 200 mg. thrice and five times daily respectively for 12 days (10 and 19 mg./kg./day).

The drug was well tolerated by 9 of the 14 patients. The remaining five complained of nausea, vomiting, and cramps in the legs. In one patient the symptoms were sufficiently serious for treatment to be discontinued.

7218 (2 : 3-Dimethylquinoxaline-1 : 4-dioxide)

7218 was less toxic than 8173 ; repeated oral doses of 250 mg./kg. could be given to mice twice daily for 12 days. A similar dose was tolerated by rats. In mice poisoned by repeated large doses of 7218 (500 mg./kg. twice daily for 16–24 days) the most prominent symptom was tonic spasm of the abdominal muscles, hind limbs, and tail, and to a less extent of the back and neck ; the fore-limbs and facial muscles were not involved. At first these spasms appeared only when the mice were disturbed. Later, recurrent tonic spasms alternating with coarse general tremor and splaying and weakness of the hind-limbs followed. These toxic signs were of interest in view of the complaints of cramps in the legs made by some patients treated with 8173. The main pathological changes in mice and rats dosed repeatedly with large amounts of 7218 occurred in the middle and deeper cortex of the adrenal ; no characteristic damage could be detected consistently in other organs. One monkey (*M. mulatta*) was dosed orally with the drug ; three doses of 200 mg./kg. were given at 3-hourly intervals on the first day ; three doses of 100 mg./kg. at similar intervals on the second day, and two doses of 100 mg./kg. on the third day. A gradual build-up in blood concentrations of drug occurred and a maximum level of 43.4 mg./100 ml. was attained one hour after the last dose. At 20 hours after the last dose the blood level was 5.6 mg./100 ml. Toxic manifestations were excessive salivation, torpidity, and clamminess to the touch. An increased tendency for the blood to clot was apparent when samples were withdrawn for analysis. The monkey finally died five days after the last dose. Two monkeys dosed thrice daily for five consecutive days with 20 mg./kg. of 7218 caused *E. histolytica* cysts to disappear from the stools. One relapsed 26 days after treatment had ceased, but the other remained clear of parasites for three months. One kitten was cured of experimentally induced amoebic dysentery, after treatment with twice-daily doses of 1, 2, 2, 5, 10, 10, and 10 mg./kg.

7218 was submitted for clinical trial against amoebiasis, but was withdrawn after reports of serious toxic effect in a trial against lymphogranuloma venereum (Alergant, to be published).

8218 (6-Chloro-2 : 3-dimethylquinoxaline-1 : 4-dioxide)

Mice survived 100 mg./kg. of the drug given orally daily for 35 days. Rats survived 16 daily oral doses of 500 mg./kg. One dog survived 26 daily oral doses of 100 mg./kg. with no ill effects and no influence on the blood picture. Another dog vomited when dosed for two days with 200 mg./kg. One monkey survived 15 daily oral doses of 500 mg./kg. It was killed after the last dose, but showed only early fatty degenerative changes in the liver. Two monkeys dosed with 200 mg./kg. (one dose and three doses respectively)

showed no ill effects, and two monkeys given ten daily doses of 20 and 40 mg./kg. respectively were likewise unaffected. The smaller doses (20 and 40 mg./kg.) were without effect on the natural amoebic infection carried by the monkeys. The monkey receiving repeated daily doses of 500 mg./kg. became free of amoebic infection after the first dose of drug.

Clinical Trial in Man.—6-Chloro-2 : 3-dimethylquinoxaline-1 : 4-dioxide was tried in five patients suffering from amoebiasis. Dr. Adams again carried out the trial, and his results may be summarized as follows:

Four patients with chronic symptomless infection were treated over periods of 6 to 14 days with doses ranging from 100–300 mg. given thrice daily. The fifth patient, who was suffering from acute amoebic dysentery, received 200 mg. of drug thrice daily for 11 days. No therapeutic effect was evident in any of the five patients, and toxic signs included cramps in the hands and feet (2), palpitation and vertigo (1), vomiting (1), sore throat (1), enlarged neck glands (1), and an urticarial papular eruption in both hands, which were blue and cold (1).

Discussion

In addition to its action against amoebiasis, quinoxaline-1 : 4-dioxide (and certain related compounds) showed activity against certain of the larger viruses (Hurst *et al.*, 1953) and certain Gram-negative bacteria (Francis, personal communication). The selection of representatives of the series for clinical trial against amoebiasis was thus governed not merely by the action of any particular substance against this particular infection, but rather by the likely overall therapeutic value as judged from laboratory results with the three types of infection—protozoal, viral, and bacterial. In addition, the toxicity and mode of absorption and excretion were taken into account. Thus the three representatives chosen for trial, 8173, 7218, and 8218, had LD50s (in mice) of 135, 360, and 500 mg./kg. respectively; whereas after oral administration (to rats) of 100 mg./kg. of drug maximum blood levels of 3.1 mg./100 ml. after 20 minutes, 4.1 mg./100 ml. after 2½ hours, and 2.0 mg./100 ml. after 1½ hours, respectively, resulted.

Experiments in rats and monkeys showed that two other substances, 2-methyl-3-ethylquinoxaline-1 : 4-dioxide (9804) and 2 : 3-diethylquinoxaline-1 : 4-dioxide (9915) had superior therapeutic properties (against experimental amoebiasis) to the three compounds actually selected for trial in man. However, as a result of the alarming toxic reactions obtained with the latter compounds, the clinical trial of further representatives of this series of compounds was considered unwarrantably hazardous.

In view of the potent anti-amoebic action of the quinoxaline oxides as measured in rats, it was disappointing to find that these compounds were comparatively ineffective against the infection in man. Although there was some indication that 8173 had a therapeutic effect on the acute phases of amoebiasis, as indicated by a favourable response in two of four cases of acute amoebic dysentery, and both cases of amoebic hepatitis, the doses used were far in excess of the dosage required to produce a therapeutic effect on rats. Thus in the successfully treated cases the daily dosages were equivalent to 16.7, 20, 19, and 10 mg./kg. respectively. In rats a total daily dosage of 5 mg./kg. was adequate. If these dosages were compared on the basis of the metabolic rate formula, the differences would be more pronounced. The toxic symptoms produced in man, e.g., nausea, vomiting, cramps, and dermatitis, could not be foreseen from the laboratory toxicological studies. Further experiments were carried out in attempts to produce compounds of greater therapeutic activity and lesser toxicity. These will be described in a later publication.

Summary

Quinoxaline-1 : 4-dioxide was found to exert a therapeutic effect against experimental amoebiasis of rats, cats, dogs, and monkeys. Certain related compounds were also active against one or more of these laboratory infections.

Quinoxaline-1 : 4-dioxide showed only weak therapeutic action when tried in man, and also gave rise to serious toxic effects. The related compound, 6-chloro-2 : 3-dimethylquinoxaline-1 : 4-dioxide, had no therapeutic effect on the disease in man, and was also toxic.

We are considerably indebted to Dr. A. R. D. Adams from whose unpublished protocols we have summarized the results of the clinical trials of 8173 and 8218.

It is a pleasure to acknowledge the technical assistance of Mr. G. H. Davies and Mr. H. H. Jones.

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SYNTHETIC AMOEBICIDES: PART III. ESTIMATION OF 6-AMINOANTHRAPYRIMIDINE IN TISSUES: METABOLISM AND EXCRETION

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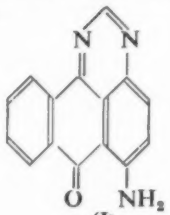
In a previous paper in this series (Jones, Landquist, and Senior, 1952) it was shown that 6-aminoanthrapyrimidine (I.C.(P) Code No. 3074) and the related compounds such as the 6-dialkylaminoalkylamines had a distinct curative action in experimental amoebiasis in young rats when tested by the method of Jones (1946). Of these, 3074, a readily obtainable substance (I), proved to be



(I)

more active than Chiniofon (7-iodo-8-hydroxyquinoline-5-sulphonic acid). It was highly active *in vitro* against *Entamoeba histolytica*, though less active than emetine in both methods of evaluation. It was well tolerated in rats, and the chemotherapeutic index was of the order of 30 to 33. Jones (personal communication), however, found that 3074 had a pronounced photodynamic action when albino rats and mice dosed with it were exposed to sunlight. In order to throw light on this effect a fluorimetric method of estimating 3074 in tissues has been devised, and a study has been made of its distribution, metabolism, and excretion in rabbits. A few tests have also been performed in rats.

EXPERIMENTAL

Estimation of 6-*Aminoanthrapyrimidine* (3074)

The substance 3074 occurs as orange yellow needles, m.p. 272–4° C. (uncorr.), when crystallized to constant melting point from xylene. It is very sparingly soluble in water (50 μg. per 100 ml. at 18° C., 250 μg. per 100 ml. at 37° C.), but rather more soluble in organic solvents, the solution in non-polar solvents exhibiting a strong green fluorescence. The greenish-yellow colour is insufficient for direct colorimetric estimation except in high concentrations. It is not practicable to diazotize the amino-group and then to form an azo compound capable of colorimetric estimation. Moreover, no colour reaction is given with either *p*-dimethylaminobenzaldehyde or β-naphthaquinonesulphonic acid.

The green fluorescence in benzene is, however, sufficiently intense to serve as a basis for analysis in the range of concentrations found in experimental animals. Extraction of tissue homogenates is very nearly complete, provided that the medium is saturated with sodium chloride; the blank values from fresh tissues are of a low order. When blood concentrations are below 10 μg./100 ml. the method is less satisfactory owing to blank interference, but this does not affect the findings recorded here, since relatively high concentrations were met with in all tissues.

Reagents

Standard Solution of 3074: 25 mg. dissolved in 40 ml. glacial acetic acid and diluted to 100 ml. with distilled water. This solution, which is stable, is diluted 1 in 250 with water to form the standard solution (1 ml. contains 1 μg.).

Benzene: Commercial benzene, the fluorescence of which has been removed by distillation through an efficient fractionating column; the first and last portions (each about 10%) are rejected.

0.2M-*Disodium Hydrogen Phosphate Solution:* Reagent purity.

Sodium Chloride: Reagent purity.

Apparatus

Spekker photo-electric absorptiometer used as a fluorimeter. Filter Green W.7. Both photocells, of 10 ml. capacity, were used.

Procedure

(1) *Standard Curve.*—A standard solution which gives a concentration of 5 μg. 3074 in 10 ml. of the benzene extract serves as a satisfactory standard against which unknown dilutions may be compared. Quantities of 10, 8, 6, 4, 3, 2, and 1 ml. of the standard solution are pipetted into 60-ml. glass-stoppered bottles and adjusted to 10 ml. with distilled water; an eighth bottle

contains water only; 5 ml. sodium phosphate solution is added to each bottle followed by excess sodium chloride; 20 ml. benzene is added by burette and the bottles shaken vigorously for 10 minutes. The benzene layer is decanted into tubes of at least 25 ml. capacity and centrifuged (1,500–2,000 r.p.m. for 10 minutes). Using these quantities, the top standard contains 5 μg. of drug in 10 ml. of benzene extract.

(2) *Estimation of Tissue Concentration.*—Blood or plasma (5 ml.) or homogenized tissue (5 ml. containing 0.5 g. of tissue) is pipetted into 10 ml. of water contained in a 60-ml. glass-stoppered bottle; sodium phosphate solution (5 ml.) is then added, followed by excess sodium chloride, and the procedure as for the standard curve is followed. It is important to use centrifuge tubes of at least 25 ml. capacity in order to obtain 10 ml. of benzene solution free from solid matter. After centrifugation, any emulsion which forms in the benzene layer is stirred with a glass rod and the tube re-centrifuged; a clear benzene layer then results with all tissues. If it is found necessary to dilute the benzene extract, distilled benzene, previously shaken with saturated brine and filtered, should be used.

(3) *Estimation of Unchanged Drug in Urine.*—Urine, or a suitable dilution (1 ml.), is pipetted into a 60-ml. glass-stoppered bottle containing sodium phosphate (5 ml.) and water (10 ml.). Benzene (20 ml.) is added and the bottle is shaken for ten minutes. The benzene layer is centrifuged before the reading is taken. Dilution of the benzene layer with centrifuged water-saturated benzene may be necessary.

(4) *Estimation of Unchanged Drug in Faeces.*—The daily output of faeces is comminuted by pestle and mortar, ground with water, and made up to 1,000 ml. A suitable portion (1 to 10 ml.) is then treated as in (3) above.

Notes.—(1) Drying the solvent after extraction or the addition of acetic acid (1%) to the extract enhances the fluorescence, but these refinements have not been adopted, for the improvements are of little practical value.

(2) Sodium phosphate is used in order to retain the fluorescent constituents of the tissues in the aqueous phase. Blank tests on blood carried out in the absence of this reagent illustrate its success in this respect. A high blank fluorescence is occasionally found in faeces, but the drug concentration is such that the accuracy of the method is little affected.

(3) The necessity for salting out with sodium chloride arises from the low recoveries otherwise obtained from blood and other tissues; urine, however, gives a satisfactory mean value of 99%. Ten recoveries from whole blood (horse) give an average of 72.5% without salt and an average of 103% with salt. Corresponding figures for rabbit are 71% and 102%.

The mean recoveries ± the standard deviation, obtained by adding various amounts of the standard solution of 3074 to homogenized rat tissues or excreta and following the analytical procedure described, were: blood, 102±3.0%; serum, 94±3.4%; liver, 82±2.6%; kidney, 83±1.2%; spleen, 94±2.0%; lung, 78±1.4%; muscle, 86±3.4%; fat, 89±1.7%; faeces, 94±2.7%; urine, 99±3.3%. About 10 analyses (9–11) were performed in each group.

Experiments on Rabbits

Determination of Blood Concentration.—Young chinchilla rabbits of either sex, weighing from 2.0 to 2.7 kg., were used. A single 100-mg./kg. dose was given orally as a dispersion in Dispersol O.G. (I.C.I.) and a sample of blood (about 5 ml.) was taken from the ears at suitable intervals (Table I). The animals were kept in subdued light to prevent skin sensitization. Urine and faeces were collected separately, at the same time; the urine was collected in flasks containing 1% mercuric chloride solution (1 ml.) and protected from light in order to minimize any possible photochemical decomposition. The urine was stored at 5° C. until required for analysis.

TABLE I

BLOOD CONCENTRATIONS AT VARIOUS INTERVALS FOLLOWING A SINGLE 100-MG./KG. ORAL DOSE IN RABBITS

Hours after Administration	Blood Concentration (μg. per 100 ml.) for Individual Animals				Mean Value
	1	2	3	4	
1·16	213	170	241	586	302
3·5	582	390	254	612	460
6·0	950	324	206	204	421
24	800	337	790	162	522
48	30	38	96	88	63
72	20	13	0	0	16
96	0	0	0	0	0

Tissue Distribution.—A rabbit receiving one 100-mg./kg. oral dose was killed after 16 hours, and an estimate was made of the total amount of free drug present in the tissues and in the lumen of the alimentary tract (Table II).

Search for Urinary Metabolites.—A preliminary examination was made of the first day's urine from a rabbit receiving the 100-mg./kg. dose. During the analytical procedure for unchanged drug it was observed that the diluted urine, after benzene extraction, was more highly pigmented than normal urine, being of a deep yellow-brown colour. A water-soluble metabolite was suspected, and qualitative tests on the diluted residual urine confirmed this view. Heating the urine with dilute hydrochloric acid followed by neutralization with excess sodium bicarbonate and subsequent extraction with benzene gave a solution with a green fluorescence similar in appearance to 3074. At this stage it was thought that the water-soluble metabolite was probably a conjugated form of 3074 which reverted to the parent substance on acid hydrolysis. The

TABLE II

DISTRIBUTION OF DRUG IN RABBIT 16 HOURS AFTER ONE 100-MG./KG. ORAL DOSE. RABBIT WEIGHT 1·7 KG.

Tissue	Weight (grammes)	Concentration (mg. per 100 g.)	Total (mg.)	Percentage of Administered Dose
Blood[1]	110	0·49	0·54	0·32
Liver	62	2·2	1·36	0·71
Kidney	14	0·63	0·088	0·05
Spleen	0·6	1·1	0·007	0·004
Lung	8·3	1·07	0·089	0·052
Muscle[1] ..	68	0·56	0·380	0·220
Fat[1]	10	0·22	0·022	0·013
Brain	8	0·95	0·076	0·045
Eyes	4·3	0·57	0·023	0·013
Stomach ..	—	—	24·60	14·50
Duodenum ..	—	—	0·67	0·40
Caecum and ileum	—	—	0·72	0·42
Large intestine ..	—	—	0·15	0·09
Faeces	None	—		
Urine[2]	120	(Free) 0·75 (Conjugated[3]) 1·98	0·90 2·38	0·53 1·4
Total ..			32·0	18·8

[1] The weights of these are estimated. [2] Including bladder contents. [3] This is described below.

following test was carried out to ascertain whether 3074 was quantitatively liberated from the water-soluble metabolite. Urine (1-ml. portions of a 1/4 dilution), which had previously been freed from unchanged drug by four extractions with equal volumes of benzene, was heated in a boiling-water bath for one hour with various concentrations of hydrochloric acid (1 ml.). Excess sodium bicarbonate was added to neutralize the acid and the usual procedure of benzene extraction and estimation was followed. Concentrations of acid ranging from 1.4N to 7.5N obviously gave complete liberation of free drug, since values between 1.8 and 2.0 mg. per 100 ml. were obtained. A concentration of 0.3N-hydrochloric acid gave only 0.6 mg. per 100 ml., and a blank estimation without acid gave 0.1 mg. per 100 ml. A further test revealed that quantitative liberation of the green fluorescent substance in the cold was caused by 6.6N-hydrochloric acid within two minutes. The following experiments were performed in order to identify this liberated metabolite.

Fourteen rabbits were each given three doses of 100 mg./kg. of 3074 on alternate days, and the urine, collected daily in the presence of mercuric chloride (approx. 1 in 20,000) was pooled, treated with $Na_2HPO_4.12H_2O$ (50 g. per litre), and stored at 0–5° C. until required. The urine collected over a period of six days measured 12.7 litres and was of a reddish-brown colour. A portion of the urine (1,000 ml.) was extracted with half its volume of distilled benzene and the yellow fluorescent extract was evaporated to 200 ml. and purified chromatographically on alumina (Spence grade 0) which had previously been freed from water-soluble alkali by extraction with boiling 5% acetic acid followed by washing and reactivation.

The experiment was conducted in subdued light and the column examined in the ultra-violet. The bulk of the dissolved substance was strongly adsorbed, but a narrow band which exhibited a yellow fluorescence was fully eluted with benzene. This fraction upon evaporation gave a trace of gummy material and was rejected. On introducing methanol (0.5% v/v gradually increased to 10%) the brown substance developed into a yellow fluorescent band, leaving a dark-brownish and non-resolvable ring at the top of the column. The yellow fluorescent eluate was evaporated to dryness, giving 20 mg. of a yellowish brown non-crystalline deposit, m.p. 260–4°. Crystallization from methanol gave 15 mg. of brown-coloured needles, m.p. 270–1°. Further crystallization gave a substance, m.p. 272–4°, which gave no depression of melting point on admixture with a pure sample of 6-amino-anthrapyrimidine, m.p. 272–4° (the melting points are not corrected). Analysis had shown that 25 mg. of unchanged drug was present in the volume of urine taken, the recovery of 15 mg. thus amounting to 60% of the theoretical.

A 10-litre quantity of the urine was freed from benzene-soluble drug by repeated benzene extraction and the concentration of hydrochloric acid was brought to normality by the addition of the concentrated acid. The mixture was then heated on a steam bath for one hour. The cooled solution was treated with excess sodium bicarbonate and extracted with benzene (two portions of 3 litres). This extract, which was washed with brine, was of a rich reddish-brown colour with a strong green fluorescence. Removal of the solvent left a dark-brown semi-crystalline material weighing 2.63 g. which could not be purified by crystallization; it was redissolved in benzene (700 ml.) and purified chromatographically on alumina. Elution with 0.5–2% methanol gave a brown diffused band bordered with a yellow fluorescence and a greyish-brown material remained at the top of the column. A narrow blue-purple fluorescent band followed the major band, but this was not investigated. The brown band was cut from the column and dried. Soxhlet extraction with methanol followed by fractional crystallization gave both needles (m.p. 269–271°; yield 0.53 g.) and prisms (m.p. 269–272°; yield 0.2 g.) which gave no depression of melting point on admixture. Further crystallization of the combined yield gave 0.55 g. of brown needles, m.p. 274°. There was no depression of melting

point on admixture with pure 6-aminoanthrapyrimidine, m.p. 272–4°. *Analysis:* Found C, 72.5; H, 3.85; N, 17.15. $C_{18}H_9ON_3$ requires C, 72.8; H, 3.6; N, 17.0%.

Analysis of the water-soluble metabolite in the volume of urine employed (*vide infra*) gave the equivalent of 1.03 g. of water-soluble drug calculated as 3074, and the recovery of 0.73 g. corresponds to 71% of this amount.

Estimation of the Water-soluble Metabolite.—The observation that the water-soluble metabolite was converted to 3074 on treatment with hydrochloric acid provided a basis for quantitative analysis, and the following technique was adopted.

Method.—The urine, or a suitable dilution (1 ml.), was pipetted into a boiling tube containing N-hydrochloric acid (10 ml.) and was mixed by means of a glass rod and immersed in a boiling-water bath for one hour. The cooled solution was transferred to a 60-ml. glass-stoppered bottle with the aid of a minimum amount of water (5 ml.) and slowly made alkaline with sodium bicarbonate of reagent purity (2 g.). Benzene (20 ml.) was added, and the procedure now followed the method for unchanged drug. The value for total drug was thus determined, and the value for water-soluble drug, calculated as the equivalent of 3074, was obtained by subtraction.

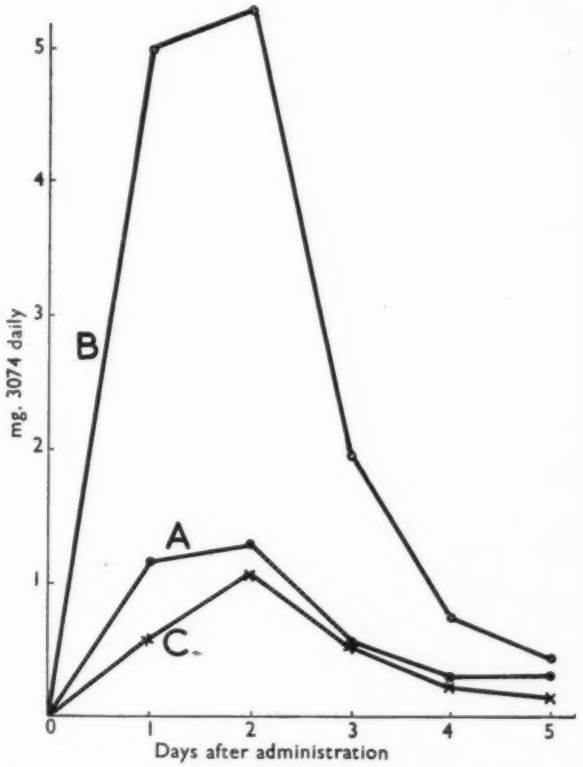


FIG. 1.—Daily excretion in four rabbits following 100-mg./kg. single oral doses of 3074, calculated as mg. per day from one 2-kg. rabbit. A is urine, unchanged drug; B urine, water-soluble drug; C is faeces, unchanged drug.

Excretion.—After a single 100-mg./kg. oral dose in four rabbits the faeces and urine were collected separately and daily. The unchanged drug in the faeces and both free and water-soluble drug in the preserved urine were determined by the methods described and are expressed in Fig. 1. The total drug excreted in the urine was equivalent to 8.6% of 3074, of which 79% was in the water-soluble form. A similar experiment with a single 200-mg./kg. dose (one animal) gave values of 7.4% and 85% respectively. Three doses of 100 mg./kg. given on alternate days (14 animals) gave the equivalent of 23.5% of 3074 in the urine, of which 81% was metabolized.

Effects of Drug Administration on Normal Metabolites

(*a*) *Sulphur Metabolism.*—One rabbit (1.77 kg.) was fed on a diet of carrot and potato (100 g. of each daily) in order to minimize the output of ethereal sulphate, and the urine was analysed for inorganic and ester sulphate by the nephelometric method (Senior, 1947). After oxidation with Benedict's sulphur reagent (Benedict, 1909) the total sulphur was estimated by this technique, thus providing, by difference, organically combined sulphur other than ester sulphate. There was no significant alteration in the sulphur metabolism.

(*b*) *Glucuronide Excretion.*—Seven rabbits receiving the previously mentioned diet were each given a single 100-mg./kg. oral dose of 3074 and the daily output of glucuronides was estimated. The naphthoresorcinol method described by Hansen, Mills, and Williams (1944) was used with modifications (Senior, 1948). Care was taken to collect the urine in flasks containing mercuric chloride solution (1 ml. of 1% solution) in order to prevent the otherwise considerable losses of glucuronide due to fermentation and to avoid a false rise which would consequently appear if the voided drug or its metabolites were bacteriostatic (Senior, 1948). The mean value taken from the daily output during the four-day periods before and after dosing was equivalent to a rise of 10.6 mg. of glucuronic acid in a 2-kg. animal. This corresponds to 8% of the drug appearing in the urine as the glucuronide.

Photochemical Decomposition of 3074

It was observed that the standard solution of 3074 which contained 0.16% of acetic acid was quite stable to light, even after several months' exposure. The presence of alkali, however, resulted in a rapid disappearance of drug when the solution

was exposed to light through window glass. A series of solutions of the same strength (8 μg./ml.) but containing different concentrations of acid or alkali were exposed to daylight for 16 hours under identical conditions. Controls were kept in the dark. Four experiments with hydrochloric acid concentrations ranging between 0.025N and normality gave recoveries of 89–101% after exposure to light, and similar results of 92–105% were given by controls kept in the dark. A neutral solution gave values of 96% and 101% respectively. A similar range of solution containing sodium hydroxide gave complete recoveries when protected from light. On exposure, however, recoveries were 7.5% (0.025N solution) and 5.6% (0.1N). With stronger alkali the recoveries were higher, being 40% at 0.5N and 52% at normality. A second series of experiments was then performed under similar circumstances but employing a range of buffered solutions. The percentage recoveries, after exposure to light, were as follows: *p*H 6.8, 95; *p*H 7.0, 27; *p*H 7.2, 15; *p*H 7.4, 12.5; *p*H 7.6, 8.0; *p*H 7.8, 8.0; *p*H 8.0, 4.5%.

The investigation was extended to the biological fluids blood, plasma and urine, and 20% dilutions were used in lieu of the inorganic solutions. A solution of 8 μg./ml. of 3074 in a 20% plasma dilution was exposed to diffused sunlight for a period of twelve hours. There was a recovery of 21%, whereas a normal recovery was given by a similar solution kept in the dark. A medium of diluted urine again revealed photochemical decomposition, for a 55% recovery was obtained after exposure for twelve hours, whereas the value was 102.5% when protected from light. On the other hand, haemolysed blood gave a full recovery (97%) on exposure to light.

The possibility of this photochemical reaction resulting in the conversion of the drug to the water-soluble form was tested by exposing a solution containing approx. 8.5 μg./ml. in 0.1N-sodium hydroxide solution to sunlight (through glass) for two hours and then determining both free and total drug. The values recorded for free and total drug were 2.0 μg./ml. and 3.0 μg./ml. respectively. A control kept in the dark gave 8.7 μg./ml. and 8.4 μg./ml. respectively.

Biological Tests on the Reduced Form

The leuco- or dihydro-form of 6-aminoanthrapyrimidine was prepared by boiling the compound with aqueous alcoholic sodium hydrosulphite solution in the presence of ammonia (I. G. Farbenind, B.P. 461,883). The product $C_{15}H_{11}ON_3$ (Code No. 6262), m.p. 272°, gave only a slight fluorescence when shaken with benzene, gave no depression with 3074, and was re-oxidized at the melting point. It was of a lower solubility than the oxidized form, to which it rapidly reverted in solution. This reduced form has been reported to be slightly less active than the oxidized form, the minimal effective dose being 50 mg./kg. for 6262 compared with 30 mg. for the compound 3074 (Jones, Landquist, and Senior, 1952).

Extraction of 6262 with boiling alcohol followed by fluorimetric analysis of the filtrate revealed that less than 0.2% of unchanged 3074 was present. A single 100-mg./kg. oral dispersion administered to albino rats exposed to sunlight gave rise to an oedematous condition followed by necrosis, though the symptoms were rather less severe than those of a similar group of rats receiving the same dose of 3074. As this was very good evidence that there was an *in vivo* oxidation to the photodynamically active 3074, two similar groups of rats were given single 250-mg./kg. oral dispersions of 3074 and 6262, and the blood concentrations (Fig. 2) and liver concentration (Fig. 3) were

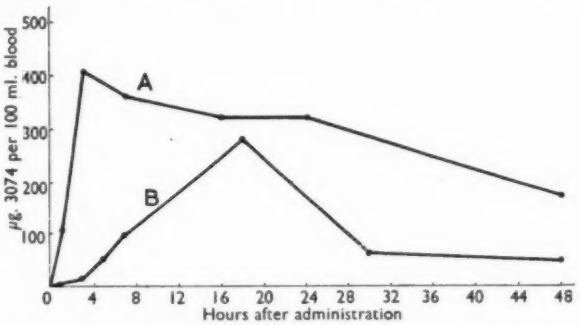


FIG. 2.—Blood concentrations of 3074 in rats following oral administration of dispersions of 3074 and 6262. Dose: 250 mg./kg. Three or four animals per point. A is 3074, B is 6262.

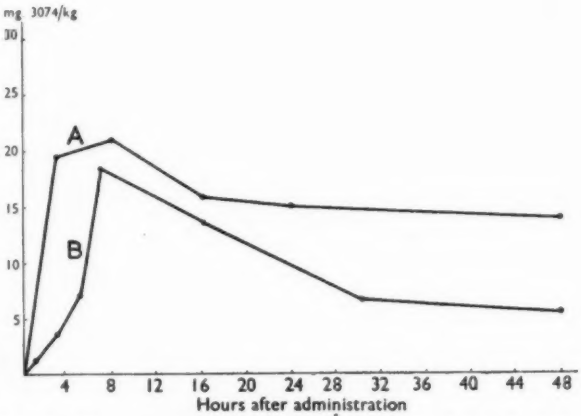


FIG. 3.—Liver concentrations of 3074 in rats following oral administration of dispersions of 3074 and 6262. Dose: 250 mg./kg. Three or four animals per point. A is 3074, B is 6262.

determined at suitable intervals, using three or four animals for each estimation. Other groups of six rats were given 100-mg./kg. doses of these substances, and the urine was analysed daily for both free and water-soluble drug for four days. The percentages of water-soluble metabolite to total drug excreted (free plus water-soluble) were 67 for 3074 and 85 for 6262.

Discussion

The substance 6-aminoanthrapyrimidine (3074) gave a high initial blood concentration in rabbit (100 mg./kg. oral), and this was maintained for at least 24 hours (Table I). Blood and liver concentrations in the rat were also of a high order (Figs. 2 and 3), and these persisted for at least 48 hours after a single 250-mg./kg. dose. Analysis of one rabbit killed 16 hours after one 100-mg./kg. oral dose revealed that 85% was absorbed (Table II), but only 4.3% of this could be accounted for. It was unfortunate that it was only in urine that it was possible to estimate the water-soluble form, for other tissues gave too high a blank reading on treatment with acid. The proportion of the water-soluble form in urine was approximately 80 to 85% of the total, and this value appeared to be approximately constant in all the samples of urine analysed. Even if the assumption be made that this ratio applies throughout the tissues the fate of the major portion of the drug would still be unknown.

It is clear that, although the dihydro- or leuco-form of the drug was not the main metabolite, it was probably the first stage in the degradation. There was no evidence that any other metabolite was present in the urine, and there was no significant rise in the excretion of the normal conjugating metabolites such as glucuronic acid or sulphur derivatives such as ester sulphate or mercapturic acid. Retention of the drug by protein binding is very unlikely. Though the anthrapyrimidine dyestuffs possess some affinity for natural fibres the 6-amino derivative is of low activity in this respect. This was reflected by the tissue recoveries, where the greatest loss due to protein binding was only 22.5% (lung), and was, of course, corrected for in the estimation. Analysis of the excreta over a period of several days confirmed that the drug was largely metabolized, for a single 100-mg./kg. dose gave rise to only 8.6% of total drug in the urine and 1.2% unchanged drug in the faeces. Likewise a 200-mg./kg. dose gave a total of 7.4% in the urine, though repeated dosing (three doses of 100 mg./kg.) gave a higher value of 23.5%.

The rise in glucuronides was probably not significant owing to the large variation in the normal excretion which occurred even on a controlled diet. The 100-mg./kg. dose was the highest which did not interfere with the food intake of the rabbit, and in view of the daily variation this was clearly too small to give a significant rise unless the major portion of the drug were to pass through to the urine and appear in the conjugated form. The observed rise in glucuronides suggested that 8% of the total drug administered was present in this form. It may have been a coincidence that this was of the same order as the finding from the fluorimetric analyses, for these were 8.6% of total drug and 6.8% of water-soluble drug. In any event the apparent rise may have been due to the excretion of other and undetected metabolites in the form of glucuronides, and it is generally not acceptable to claim that a metabolite appears as a glucuronide unless it has been isolated as such. The view is held, nevertheless, that the water-soluble form is solubilized by conjugation in some manner, probably as the glucuronide, and that it is hydrolysed on acidification, the resulting 6-aminodihydroanthrapyrimidine then undergoing aerial oxidation to 3074. Good evidence in favour of this was obtained from the synthetic 6-aminodihydroanthrapyrimidine (6262), which was only very sparingly soluble in water and which reverted to the oxidized form in solution.

The query arises as to which form of the drug was the active amoebicide. That the active substance was in no way associated with the photochemical decomposition has been clearly demonstrated by Jones (private communication), who showed that 3074 was equally curative in rats kept in the dark. Active metabolites were an improbability, for 3074 exhibited high *in vitro* activity (Jones, Landquist, and Senior, 1952), and yet this does not preclude the possibility that the drug may have previously been reduced by the medium. The synthetic reduced form 6262 was very nearly as active as 3074, but reference to Figs. 2 and 3 will reveal that the substance was re-oxidized on absorption and that there was consequently an appreciable blood and tissue concentration of 3074, though these values were considerably lower than those resulting from a similar dose of 3074. The two forms of the drug gave comparable urine analyses. The main evidence in favour of 3074 being the active amoebicide was that it was more active when given in this, rather than the reduced form, and that the blood concentration of the oxidized form was higher under these circumstances.

The photodynamic effect was obviously associated with the presence of the green fluorescent 3074 in the exposed parts. The appreciable blood concen-

tration illustrated its presence there, and *in vitro* tests revealed that the *p*H of the blood (*p*H 7.3–7.5) fell within the range required for photochemical decomposition. The compound was unaffected by alkali in the dark, was decomposed at *p*H 7 by light passing through window glass, and was stabilized by acid, for it was unaffected by light at *p*H 6.8. Complete recovery was obtained from diluted blood exposed to light, whereas in a medium of diluted plasma there was a considerable loss on exposure. This apparent anomaly is readily explained, for the intense coloration caused by the haemolysis would inevitably absorb most of the available light energy. The circumstances are very different *in vivo*, for the continual movement of the peripheral circulation ensures that the layers of blood or plasma are adequately exposed. Losses also occurred in urine on exposure to light, but these have no bearing on the accuracy of the determinations, for precautions were taken to collect the urine in shielded flasks.

The tissue reactions resulting in the oedematous condition and the photochemical decomposition of 3074 were thus shown to be taking place concurrently and in the same tissues. Whilst it is possible that the oedematous condition may be caused by the photochemical decomposition product, strong evidence against this is provided by naturally occurring fluorescent substances, or fluorescent metabolites of plant constituents, which have been observed in the blood of animals suffering from photogenic diseases such as hypericism, fagopyrism, and tribulosis (Brockmann, 1952). The active substances are of different chemical types and as a consequence bear no resemblance to 3074. There is little chance that this uncommon phenomenon would be caused by their unrelated photochemical decomposition products. Other synthetic fluorescent substances have long been known to exhibit this activity, and probably the first reported was eosin (Raab, 1903). Amongst medicinal compounds taken internally the fluorescent acriflavine (Jauson and Marceron, 1925) and riboflavin (Boehm, 1941) have given rise to photodynamic effects. As early as 1904 it was claimed that all substances which produce photodynamic action are fluorescent (Tappeiner and Jodlbauer, 1904), and no author has since demonstrated the phenomenon and at the same time proved the absence of a fluorescing substance from the affected parts.

Summary

1. A fluorimetric method has been devised for the estimation of 6-aminoanthrapyrimidine (3074) in animal tissue and excreta, and satisfactory recoveries have been obtained over the range of concentrations found in the rabbit and in the rat.

2. When administered orally to these animals as an aqueous dispersion, the drug was rapidly absorbed and gave a blood concentration ranging between 200 and 500 μg. per 100 ml., and this was maintained for at least 24 hours. The drug was rapidly metabolized, and only 1.5% of the original drug (100 mg./kg. dose) was detectable in the tissues of the rabbit after 16 hours.

3. Only a small proportion of the drug reappeared in the urine (2–4%), but a water-soluble form of the leuco-derivative was also present, and this was quantitatively reconverted to 3074 by treatment with dilute acid. The ratio of the two forms was roughly constant, and approximately 80% was in the reduced state.

4. Comparison of this water-soluble form with the synthetic leuco-derivative (6262) suggested that it was probably conjugated with a normal metabolite such as glucuronic acid. Oral administration of 6262 resulted in a partial oxidation to 3074 which was detectable in the blood, liver, and urine.

5. The photodynamic effect of 3074, in common with other synthetic and naturally occurring substances, was associated with its intense fluorescence, and the photochemical decomposition of the drug *in vitro* took place over the same *p*H range as that obtaining *in vivo*.

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THE THERAPY OF EXPERIMENTAL PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM (INGUINALE)

II. THE ACTIVITY OF QUINOXALINE-1 : 4-DIOXIDE AND SUBSTITUTED AND RELATED COMPOUNDS, WITH A NOTE ON THE MORPHOLOGICAL CHANGES INDUCED IN LYMPHOGRANULOMA VIRUS BY THESE COMPOUNDS AND BY ANTIBIOTICS

BY

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In previous communications (Hurst, 1948; Hurst, Peters, and Melvin, 1950) we have recorded the therapeutic action of Nitroakridin 3582 and compared those of penicillin, chloramphenicol, aureomycin, and terramycin against psittacosis and lymphogranuloma venereum in mice or in developing chick-embryos. For the past eight years we have used these experimental infections (among others) in routine screening-tests of synthetic organic compounds for antiviral properties, and during this time have encountered a number of quite dissimilar chemical types each exhibiting some degree of activity against these particular infections. The most active compounds were quinoxaline-1:4-dioxide and various substituted derivatives, the best of which closely approached aureomycin in therapeutic potency. Alergant (1953) has confirmed this action in human lymphogranuloma, though toxic side-reactions rule out the possibility that the compounds will be useful additions to the pharmacopoeia. The quinoxaline oxides also manifest anti-amoebic and antibacterial properties (Jones, Landquist, and Stewart, 1953; McIlwain, 1943; Francis, 1953).

METHODS

The strains of virus used, the general method of working, and the method of computing 40% end-points (not 40% mortalities) in titrations of virus are sufficiently described in our earlier publications. When this work first began aureomycin was unknown, and in the earlier experiments we had no suitable standard of therapeutic activity. When later aureomycin became available in strictly limited quantities, we used it as a standard of reference in all experiments, giving a single dose of 1 mg. to eggs or 2 mg. twice daily to mice. Although when the antibiotic was more freely available it appeared that these were perhaps not quite the optimal doses (see Hurst, Peters, and Melvin, 1950), for the sake of consistency we decided to retain them throughout this investigation. Our experience over more than a hundred experiments, however, strongly suggests that the earliest samples of aureomycin were less effective than those issued somewhat later, so that in fact we did not achieve perfect consistency over the years, though we believe that we have done so for the greater part of the time. The largest amounts administered of other compounds were usually the "maximal tolerated doses" defined as follows: (*a*) for the mouse, as that quantity which, given twice daily (once on Saturday and Sunday) over a period identical with that in the chemotherapeutic test, caused no deaths and allowed the animals to attain a final weight not less than 90% of that of the controls; (*b*) for the egg, as that quantity which killed no embryos within five days of a single injection into the yolk-sac on the seventh day of incubation. At this point it may be noted that on many occasions we found that the maximal tolerated dose did not necessarily give results as favourable as a rather smaller one.

RESULTS

The Action of Quinoxaline-1 : 4-dioxide

Twice-daily oral doses of 0.5 mg. quinoxaline-1:4-dioxide per 20 g. greatly reduced mortality in groups of 30 mice infected intraperitoneally with psittacosis or lymphogranuloma virus. Thus in

nine experiments 25 to 30 control mice died as against 0 to 5 treated mice, while in three others the deaths among treated animals numbered 10, 10, and 11; the mean period of survival of mice ultimately dying was very materially lengthened in the treated groups. These results obtained whether treatment began 4 hours before infection with virus or 24 to 72 hours after, and an appreciable effect on mortality followed even when treatment was deferred until some animals of the group were moribund. Daily titrations of virus in the pooled spleens of six treated and six control mice showed this therapeutic effect to be associated with a tenfold to a hundredfold reduction in the growth of virus, though *in vitro* the drug did not inactivate even weak concentrations of virus within a period of 24 hours.

The optimal period of medication was 7 to 12 days; dosing for only 4 days gave inferior results, while no advantage accrued from prolonging treatment to 18 days. Twice-daily dosing was more effective than was exhibition of larger doses once daily or once every two days. Doses of 0.1 mg./20 g. were much less successful, as were usually the larger doses of 1 and 2 mg./20 g. Treatment was successful against even massive infecting doses of virus; indeed on several occasions we noted the curious phenomenon that, when treatment started 4 hours before infection with virus, it was relatively more effective against large than against smaller doses of virus. Table I illustrates this

TABLE I

EFFECT OF DOSE OF VIRUS ON THE OUTCOME OF TREATMENT OF PSITTACOSIS WITH QUINOXALINE-1:4-DIOXIDE

Virus was injected intraperitoneally four hours after the first dose of drug. Drug was given orally in doses of 0.5 mg./20 g. twice daily for 12 days. The animals were observed for 46 days. The figures in parentheses are the mean periods of survival of fatal cases in days

Infecting Dilution of Virus	Mortalities in Groups of 30 Mice			
	Experiment 1 (KLG Virus)		Experiment 2 (Ps Virus)	
	Treated	Untreated	Treated	Untreated
10^{-5}	2 (17·0)	3 (19·3)	8 (21·6)	9 (24·3)
10^{-7}	3 (15·0)	5 (10·8)	16 (23·1)	22 (10·1)
10^{-6}	3 (14·6)	7 (10·4)	16 (27·6)	23 (9·6)
10^{-4}	3 (17·6)	24 (8·8)	15 (29·2)	25 (8·8)
10^{-2}	8 (21·3)	25 (8·2)	12 (21·8)	28 (4·3)

phenomenon with psittacosis virus. It is probably explained in terms of the greater antigenic stimulus provided by the larger dose of virus during the period when its growth was largely suppressed by the drug; the smallest doses of virus evoked no immunity, and, as virus was not completely eradicated by therapy (see below), in the absence of immunity it still led to death of the more susceptible animals after dosing ceased.

Passage at about the 45th day of the spleens of mice recovered from one or other infection showed that 166 of 193 (86%) carried virus. In a long-term experiment in which 45 of 82 treated survivors from an initial group of 100 infected with psittacosis were killed at intervals between the 45th and 268th days, virus was still present in about three-quarters of the animals (74%). The 37 mice surviving to the 277th day were reinoculated with virus. Twenty-nine (78%) remained well, while the remainder developed psittacosis and died as did each of six previously uninfected controls. The close correspondence between the number of mice proving immune to reinoculation and that of animals carrying virus in the spleen is unlikely to be coincidental. The observations suggest that many clinically recovered mice carry virus for the rest of their lives. Nevertheless, 24 fresh mice introduced into the cages containing these mice, and left there from the 34th to the 277th day, developed no sign of disease; at the end of the period of contact they were not immune. The mice carrying virus were not therefore contagious for others. Meyer and Eddie (1951) have recorded analogous behaviour in human psittacosis.

Combined therapy of infected mice with quinoxaline-1:4-dioxide and aureomycin or procaine-penicillin usually gave results inferior to those with one drug alone.

A single dose of 0.25 mg. quinoxaline-1:4-dioxide given into the yolk-sac of developing eggs two hours after infection with the virus of lymphogranuloma venereum prolonged the mean period of survival of the embryos by about 50%. Doses of 0.1 and 0.5 mg. were rather less effective.

Mice infected intramuscularly with the virus of herpes febrilis or louping-ill, intracerebrally with mouse-adapted human poliomyelitis, or intranasally with influenza A received no benefit from medication with quinoxaline-1:4-dioxide.

Although thus possessing very marked activity against the largest viruses, quinoxaline-1:4-dioxide appeared to be therapeutically inferior to aureomycin. This opinion was based not so much on the figures for mortality as on the better condition of the aureomycin-treated mice during the active stages of the infection, and on their greater mean weight at the end of the experimental period. In both infected and non-infected animals the quinoxaline compound caused some retardation of growth, and an appreciable difference in weight from undosed controls still existed six and a half months after treatment ended. Among the substituted quinoxaline dioxides we have found compounds apparently free from this disadvantage and

at the same time possessing rather greater therapeutic activity than quinoxaline dioxide itself.

Examination of Substituted Quinoxaline-1:4-dioxide and Related Compounds

We have examined 93 compounds (Landquist and Stacey, 1952; Landquist, 1951; Landquist, 1952) for activity against lymphogranuloma in the chick-embryo, and 46 of these for activity against this disease and often psittacosis in the mouse. Groups of 18 eggs received a single dose of compound into the yolk-sac two hours after injection of virus. Groups of 30 mice received a large intraperitoneal dose of virus, and 48 to 72 hours were allowed to elapse before treatment started, usually by oral dosing twice daily for 12 days. Table II summarizes the results obtained with 10 of these compounds as typical of the observations made in the larger series. The results are expressed as percentages of the effect produced by a standard dose of aureomycin in the same experiment. Thus, with mice, if only 2 untreated controls survived while 28 of the aureomycin-treated animals did so, representing a saving of 26 lives, and in a quinoxaline-treated group 15 survived representing a saving of 13 lives, the percentage effect was held to be 50.

TABLE II

SUMMARY OF THE THERAPEUTIC ACTIVITY AGAINST PSITTACOSIS AND LYMPHOGRANULOMA OF 10 SUBSTITUTED QUINOXALINE-1:4-DIOXIDES

The entries in the Table (except in the last column) represent the therapeutic effects of the compounds expressed as percentages of that produced by aureomycin in the same experiment. The exact method of calculation is described in the text. The standard dose of aureomycin in eggs was 1 mg., in mice 2 mg. orally twice daily for 12 days. Figures in parentheses are means. The entries in the last column are the percentages of surviving mice carrying virus in the spleen at about the 40th day. R = quinoxaline-1:4-dioxide

Compound	Dose in mg.	LGI Virus in Chick-embryo, % Effect	LGI Virus in Mouse, % Effect	Ps Virus in Mouse, % Effect	% Surviving Mice Carrying Virus*
7331 2-Methyl—R	1·5	67	—	—	—
	0·5	99	45, 40 (43)	11	—
	0·1	—	5	0	—
8183 6-Methyl—R	5	—	104 88 (96)	50, 100 (75)	—
	1·5	49, 50 (50)	100	100, 96 (98)	92
	0·5	68, 59 (64)	50	24, 40 (32)	—
	0·2	73, 25 (49)	—	—	—
7218 2:3-Dimethyl—R	5	53, 34, 42 (43)	84, 125 (105)	54	38
	1·5	—	117, 95, 91, 125, 111, 104, 100, 125 (109)	24	67
	1	48, 64, 61 (58)	—	—	—
	0·5	86, 69, 122, 109, 83, 72, 108, 86, 66 (89)	81, 105, 121, 100 (102)	40	100
	0·2	37	37, 43 (40)	—	—
	0·1	—	4	—	—
9630 2-Methyl, 3-amyl—R ..	10	41, 39 (40)	30	—	—
	5	30	—	—	—
	2	2	25	—	—
8218 6-Chloro, 2:3-dimethyl—R	5	—	96, 88 (92)	46, 48 (47)	—
	1·5	—	113, 115 (114)	16	—
	0·5	44, 51 (48)	104	36	—
	0·2	33, 63, 44 (47)	—	—	—
	0·1	24	—	—	—
9677 6-Bromo, 2:3-dimethyl—R..	5	—	83, 105 (94)	100	64
	2	43	—	—	—
	1	30, 47 (39)	—	—	—
	0·5	88	—	—	—
8811 5-Methoxy—R	1	—	92, 113 (103)	93, 92 (93)	87
	0·5	34, 38 (36)	—	—	—
	0·2	46, 59, 53 (53)	113	80	—
	0·1	57	25	16	—
	0·05	33, 39 (36)	—	—	—
9265 5-Methoxy, 2:3-dimethyl—R	5	5, 5 (5)	—	—	—
	2	—	−16, −4 (−12)	50, 24 (37)	—
	0·5	3	−7	−12	—
9275 6-Methoxy, 2:3-dimethyl—R	5	37, 27 (32)	121	100	65
	1·5	—	67	52	—
	1	0, 5 (3)	—	—	—
9592 6-Acetamido—R	5	—	110	—	—
	2	41, 40, 68 (50)	85	88	—
	1	55	—	—	—
	0·5	51, 47 (49)	11	36	—

* Virus carriers among mice treated with aureomycin = 65%.

With developing eggs a similar calculation was based upon the mean period of survival of the embryos in days after infection ; embryos surviving until the eggs were opened 13 days after infection (20th day of incubation) were deemed to have survived for one additional day.

From the data summarized in Table II and the full series of observations we reached the following conclusions:

(i) The standard doses of aureomycin were not quite the best which could have been chosen. Larger doses gave slightly superior results, but were not possible in the earlier stages of the work, when great economy was needed in the use of the antibiotic. As explained previously, for the sake of consistency we adhered to these doses when the antibiotic became more freely available.

(ii) Of the quinoxaline oxides the largest doses used were near the maximum tolerated as previously defined. The relation between the tolerated dose for mice and that for chick-embryos varied. Often, weight for weight, mice tolerated larger doses, and of course in mice these doses were repeated. The reverse obtained with 7331, 9265, and some other compounds. The best therapeutic results did not necessarily follow administration of the largest amount tolerated.

(iii) When activity was expressed in terms of that of aureomycin under identical conditions, few compounds were as effective against lymphogranuloma in the egg as against the same disease in the mouse ; in this respect 7331, 9630, and a few other compounds were exceptional. Allowing for the considerable margin of experimental error inherent in these observations, fair agreement usually existed between the relative order of activity of various quinoxaline oxides in eggs and in mice. Activity against psittacosis in the mouse usually corresponded with that against lymphogranuloma in the egg rather than lymphogranuloma in the mouse. However, 8183, 8811, 9275, 9592, and 9677 were highly active against both diseases in the mouse ; 9265 was exceptional in showing some activity against psittacosis in the mouse and none against lymphogranuloma in either mouse or egg, while 7331 showed good activity against lymphogranuloma in the egg, moderate activity against lymphogranuloma in the mouse, and negligible activity against psittacosis in the mouse.

(iv) Against lymphogranuloma in the mouse, the best compounds exhibited activity roughly comparable with that of aureomycin ; as with the antibiotic, activity was evident whatever the route of inoculation of virus—intraperitoneal, intranasal, or intracerebral. While a large amount (10 mg.) of aureomycin (or an associated diluent in the capsules—cf. Cabasso, Moore, and Cox, 1952) inactivated 10 LD50 psittacosis virus *in vitro* in less than 2 hours at room temperature, a similar dose of 7218 did not do so within 6 hours. Yet, as seen from Table III, 7218 was highly active in restraining or suppressing growth of virus in the mouse. Despite this marked effect during the period of medication, virus often reappeared when treatment ceased (cf. Hurst, Peters, and Melvin, 1950), and as seen from Table II many animals carried virus in the spleen at about 40 days after infection ; the proportion so doing appeared to diminish with increased dosage of the drug. The apparent absence of virus during therapy was probably not due to the masking effect of drug or a metabolite, since serum or splenic suspensions from treated uninfected animals had no power to inactivate even small quantities of virus when the two were mixed and injected into fresh mice.

(v) Against equine encephalomyelitis, herpes febrilis, and ectromelia in mice several substituted quinoxaline dioxides had no action. Furthermore, Dr. M. G. P. Stoker very kindly tested 7218 against

TABLE III

PSITTACOSIS VIRUS: DAILY TITRES IN THE SPLEENS OF MICE TREATED OR UNTREATED WITH 7218 OR AUREOMYCIN

Virus was inoculated intraperitoneally. At the times stated the pooled spleens of 6 mice were titrated and the dilution of virus giving 40% end-point in the passage-mice was derived by the method described elsewhere (Hurst, Peters, and Melvin, 1950). The $\log_{10}$ dilutions are given below as positive quantities. N.V. = no virus detected

Infecting Dilution of Virus	Treatment	Treatment Begun	Days After Infection				
			1	2	3	4	7
10^{-5}	None	—	2·5	4·1	5·9	5·7	5·2
	7218 5 mg./20 g. orally twice daily	4 hours before virus	N.V.	N.V.	N.V.	N.V.	N.V.
10^{-3}	None	—	2·7	6·5	6·8	7·0	4·9
	7218 5 mg./20 g. as above	4 hours after virus	1·5	N.V.	N.V.	N.V.	N.V.
	7218 1 mg./20 „ „ „	„ „ „ „	2·3	5·5	4·0	3·2	1·1
10^{-4}	None	—	3·1	5·0	6·0·	6·9	6·5
	7218 5 mg./20 g. as above	4 hours after virus	0·1	N.V.	1·0	N.V.	N.V.
	Aureomycin 2 mg./20 g. as above	„ „ „ „	N.V.	N.V.	1·5	0·2	N.V.

R. burneti infection in the chick-embryo and found that it showed no useful activity, while we ourselves could demonstrate no activity against *Toxoplasma* in mice.

In view of the foregoing results it was disappointing that Alergant (1953), while confirming the therapeutic activity in man, found it inseparable from unpleasant toxic side-reactions. At various times and in various places† quinoxaline-1:4 dioxide itself, 7218, or 8218 have been tried clinically in man and have always shown these toxic effects when given systemically in adequate therapeutic doses (see also Jones, Landquist, and Stewart, 1953). Of the toxic effects, dermatitis has not been observed in animals, but tonic spasms of the abdominal muscles, hind limbs and tail, and to a less extent of the back and neck, may be caused in mice by proportionately very much larger doses than are responsible for symptoms in man. Although the toxicity of different derivatives varies materially, so also does therapeutic activity, and in experiments on mice we have never met with any indication that the therapeutic ratio of a particular compound was more favourable than that of the best in Table II.

We shall not present detailed results in respect of the other compounds examined, mostly against lymphogranuloma only but sometimes against psittacosis in addition. A classified list follows, indicating the substituent group in each together with the degree of their activity in eggs (E) or mice (M). Compounds showing 80–120% of the activity of aureomycin were considered to be highly active (++), 25–80% as moderately active (+), below 25% as slightly active or inactive (0). The compounds marked with asterisks were examined on two to four occasions and with these the classification is beyond doubt. Because of experimental error it is possible that repeated examination of some of the others might have caused them to be moved into a neighbouring class; it is certain, however, that a slightly active compound would not have appeared as highly active and vice versa.

Monosubstituted Quinoxaline-1 : 4-dioxides

10,856	2-Ethyl	E++,M+*
11,861	2-Ethoxymethyl	E+
14,026	2-Acetoxymethyl	E++
8333	6-Chloro	E+,M++*
9553	6-Bromo	E+*
10,309	6-Amino	E++,M++*
8358	6-Methoxy	E++,M+
10,203	6-Ethoxy	E+,M+
12,563	6-*n*-Butoxy	E+
12,371	6-β-Methoxyethoxy	E++*

Disubstituted Quinoxaline-1 : 4-dioxides

9804	2-Methyl-3-ethyl	E+,M++*
9915	2:3-Diethyl	EO,M++*
10,323	2-Methyl-3-*n*-propyl	EO,M++*
10,931	2-Ethyl-3-*n*-propyl	EO,M++*
9833	2:3-Di-*n*-propyl	EO,MO*
8175	2:3-Diphenyl	E+,MO*
12,248	2-Methyl-3-carbethoxy	E+
11,160	2-Methyl-3-β-ethoxyethyl	E+,M++
12,922	2:3-Di(hydroxymethyl)	E+
12,879	2:3-Di(iodomethyl)	EO
12,911	2:3-Bis(dimethylaminomethyl)	EO
12,880	2:3-Di(anilinomethyl)	EO
12,634	2:3-Di(piperidinomethyl)	EO
13,079	2:3-Di(β-piperidinoethyl)	EO
12,818	2:3-Di(bromomethyl hexamine compound)	E+
12,963	2:3-Di(trimethylammonium methyl bromide)	EO
12,964	2:3-Di(pyridinium methyl bromide)	EO
9947	6:7-Dimethyl	E++,M++*
9270	6:7-Dichloro	E+*
10,389	6-Chloro-7-methyl	E++,M+*
10,791	6-Bromo-7-methyl	E++,M+*

Substituted 2 : 3-dimethylquinoxaline-1 : 4-dioxides

8219	6-Methyl	EO,M++*
8986	6-Nitro	EO,MO
11,306	6-Trifluoromethyl	E+,M++*
9378	6-Cyano	E+*
10,685	6-Carbethoxy	E+
10,628	6-Carbamyl	E+
10,879	6-γ-Piperidinopropylcarbamyl	E+
11,070	6-Methylsulphonyl	E++*
11,736	6-Ethylsulphonyl	E+*
12,373	6-(2′-Methoxyethyl)-sulphonyl	E+
11,971	6-Carboxymethylsulphonyl	E+
10,226	6-Amino	E+,MO
9586	6-Acetamido	EO,MO
10,821	6-*p*-Toluenesulphonamido	EO

† We are greatly indebted to our colleague Dr. G. I. Robertson, at present in Nairobi, and to Drs. A. R. McKelvie (Nairobi), R. Lewthwaite (Colonial Office), A. H. Lowther (Kuala Lumpur), F. Maxwell Lyons (Giza), and A. O. Ross (Liverpool) for help with one or other aspect of the clinical trials.

11,031	6-*p*-Aminobenzene-sulphonamido	EO
10,328	6-(2′-Amino-1′:6′-dimethyl-pyrimidinium-4′-amino)iodide	E+*
11,788	5-Hydroxy	EO
10,513	6-Hydroxy	EO
10,234	6-Ethoxy	EO,MO*
12,564	6-*n*-Butoxy	EO
12,253	6-(2′-Hydroxyethoxy)	EO*
12,370	6-(2′-Methoxyethoxy)	EO
12,250	6-(2′-Acetoxyethoxy)	EO
9920	6:7-Dimethyl	E+,MO
10,548	6-Chloro-7-methyl	E++,M++*
11,147	6-Bromo-7-methyl	E+,M+
9629	6:7-Dimethoxy	EO,MO

Phenazine Di-N-oxide and Related Compounds

2781	Phenazine di-N-oxide	EO,MO
11,975	2-Piperidinophenazine di-N-oxide	EO
10,000	1:2:3:4-Tetrahydrophenazine di-N-oxide	E+,M++*
10,501	6-Methyl-1:2:3:4-tetrahydrophenazine-di-N-oxide	EO,M+
10,819	6-Chloro-1:2:3:4-tetrahydrophenazine-di-N-oxide	E+,M++
10,965	6-Methoxy-1:2:3:4-tetrahydrophenazine-di-N-oxide	E+,MO
12,833	2:3-Pentamethylenequinoxaline-1:4-dioxide	EO

Diquinoxalyl Tetra-N-oxides

11,078	1:2-Di-(6′-quinoxalyl-oxy)ethane tetroxide	E+,MO*
10,367	1:3-Di(6′-quinoxalyl-oxy)propane tetroxide	EO
11,074	1:5-Di-(6′-quinoxalyl-loxy)pentane tetroxide	E+*
10,368	1:3-Di-(2′:3′-dimethyl-quinoxalyl-6′-oxy)propane tetroxide	EO
11,073	1:5-Di-(2′:3′-dimethyl-quinoxalyl-6′-oxy)pentane tetroxide	EO
11,734	1:2-Di-(2′:3′-dimethyl-quinoxalyl-6′-sulphonyl)-ethane tetroxide	EO
10,514	2:3:2′:3′-Tetramethyl-6:6′-diquinoxalyl-sulphone tetroxide	E+,M+

Miscellaneous Compounds

8271	Quinoxaline-l-oxide	E+,MO*
9989	2-Methyl-3-acetylquinoxaline-1-oxide	EO
11,954	2:3-Dimethylquinoxaline-6-sulphonic acid mono-N-oxide	EO
9591	1-Methylquinoxal-2-one-4-oxide	EO
9502	1-Methyl-3-hydroxy-quinoxal-2-one-4-oxide	EO
10,565	1-Methylbenz-1:2:3-triazole-3-oxide	EO,MO
12,514	2:3-Dimethyl-l-methoxyquinoxalinium-4-oxide *p*-toluenesulphonate	EO
13,489	1-Methylquinoxalinium-4-oxide iodide	EO
8379	2:3-Diphenylpyrazine-1-oxide	E+,MO
8378	2:3-Diphenylpyrazine-1:4-dioxide	EO,MO
11,232	2:5-Diphenyl-3:6-dimethylpyrazine-1:4-dioxide	EO

These results warrant few conclusions regarding the relation between chemical structure and biological activity. However, in view of the importance of the $-NO_2$ group in determining antiviral activity in some acridine derivatives (Eaton, van Allen, and Wiener, 1947), it is worth drawing attention to the inactivity of 6-nitro-2:3-dimethylquinoxaline-1:4-dioxide (8986) when a number of closely related compounds in which the $-NO_2$ group was replaced by Cl, Br, CF_3, etc., showed considerable activity.

Miscellaneous Observations

Combined Treatment with Vitamin K and a Substituted Quinoxaline Dioxide.—It may be recalled that McIlwain (1943) prepared quinoxaline oxides as potential vitamin K antagonists, although he did not establish the fact that such antagonism exists. When we treated mice infected with lymphogranuloma with both 7218 and maximal tolerated doses of "Synkavit" (Messrs. Roche Products, Limited) or other preparations of synthetic vitamin K, the quinoxaline dioxide still exerted its usual therapeutic effect. Given alone, the vitamin did not itself affect mortality.

Morphological Changes in the Virus of Lymphogranuloma Treated with Antibiotics or a Substituted Quinoxaline Dioxide.—We examined

histologically the yolk-sacs of over 90 embryos sacrificed at various times after treatment with single doses of crystalline penicillin G (sodium salt) ranging from 250 to 4,000 units, of aureomycin from 0.025 to 2 mg., or of 7218 from 0.2 to 1.5 mg. The tissues were fixed in Zenker with acetic acid and sectioned at 2 μ; in staining we obtained much better results with Giemsa than by Noble's method (Yanamura and Meyer, 1941), which differentiated less clearly the virus from normal constituents of the yolk.

Rake and Jones (1942) described in detail the development of lymphogranuloma virus in the yolk-sac, and it would be superfluous here to duplicate their admirable account. Suffice it to say that, with the doses of virus used in our experiments, infected cells containing virus particles of 0.5 to 2 μ in diameter ("initial bodies") were readily detected at 16 hours when the first observation was made. Thereafter elementary bodies appeared and, together with a proportion of the larger forms, increased rapidly in number as the colonies of virus enlarged greatly. More and more cells became infected, until at 72 hours practically every cell lining the yolk-sac showed larger or smaller colonies. Soon afterwards signs of impending disintegration were evident, and abundant elementary bodies and colonies of virus escaping from ruptured cells lay free in the yolk. A typical smallish colony of virus is depicted in Fig. 1.

The morphological appearances in embryos treated with penicillin closely resembled those described by Weiss (1950) in eggs infected with the viruses of feline and murine pneumonitis. Such differences as were noted were probably due to the different experimental conditions; Weiss applied treatment 3 days after inoculation of virus, which thus had appreciable time in which to develop normally, while we injected drug only 2 hours after infection. However, since our work was completed before the publication of Weiss's paper and we have not had the opportunity of repeating it under other conditions, we cannot exclude the possibility of slight variations in the response to antibiotics of the different larger viruses. With single doses of 250 or 1,000 units, the appearance of virus in cells lining the yolk-sac was not appreciably retarded, and only slightly after 4,000 units. During much of the period of development virus existed exclusively in the form of large plaques ranging from 2 μ to as much as 6 μ in diameter, with a preponderance of the larger sizes; these usually stained a deep purplish blue with Giemsa, but sometimes a paler blue, and a few showed a vacuolated structure. In some instances a group of intensely coloured plaques seemed to be embedded in a paler blue matrix. The size of these plaques was not influenced by the dose of antibiotic within the range used. With the larger doses of penicillin infection of new cells occurred less readily, so that at 4 days less than 10 to 20% were visibly affected and advanced infection was retarded until the 5th or 7th day, when perhaps the effect of the antibiotic was wearing off. Although now the yolk-sac was visibly disintegrating, most of the virus still existed as large plaques, though a few of the masses had divided into elementary bodies or forms rather larger than these. Some of the plaques free at this time in the yolk stained very poorly and were possibly degenerate. Fig. 2 illustrates the state of affairs at 96 hours.

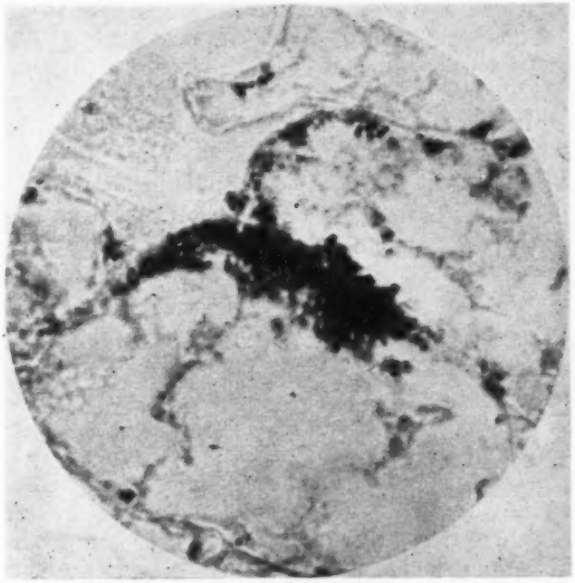

FIG. 1.—Infection of the yolk-sac with the virus of lymphogranuloma; a smallish colony of virus at 48 hours. Giemsa's stain. Magnification, ×1,000 diameters.

The picture with aureomycin was quite different. A dose of 0.025 mg. only slightly retarded the progress of viral growth and the ultimate dissolution of the yolk-sac; the morphological appearances were identical with those in untreated embryos. Larger doses (0.1 and 0.5 mg.) markedly delayed the appearance of recognizable virus, and moderately heavy infections were not present until the 5th to 7th day. In some cells, particularly at intermediate stages, virus tended to occur exclusively in plaques rather larger than normal, while in other cells at this stage and in nearly all later on the normal appearances were seen. Development of large forms was much less pronounced than portrayed by Gogolak and Weiss (1950) in feline

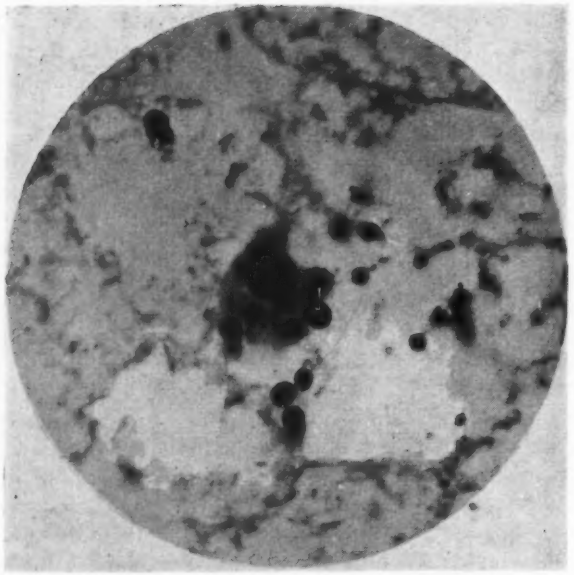

FIG. 2.—Effect of treatment with 1,000 units of penicillin; a virus colony 96 hours after infection. The largest plaque is nearly 5 μ in diameter. Some plaques stain less deeply than others, and around some of the larger group there are suggestions of a still paler matrix which do not appear to be wholly due to an optical effect. Staining and magnification as in Fig. 1.

pneumonitis virus treated with aureomycin under rather different conditions. The gigantic forms seen with penicillin were absent. In a few yolk-sacs treated with the still larger dose of 2 mg. no virus was detected histologically on the 7th or 10th days, though from approximately one-third of the chickens hatched from eggs so treated virus could be recovered by passage in animals.

Appearances in embryos treated with 7218 closely resembled those in the preceding group. With a dose of 0.2 mg. dissolution of the yolk-sac was retarded by a day or two, with larger doses proportionately longer. Virus occurred mainly in the normal forms, and giant forms were not encountered with the doses of compound used.

It would seem, therefore, that on the basis of morphological changes induced in the virus penicillin differs considerably in its action from aureomycin and the quinoxaline oxides.

Attempts to Engender Drug-resistance in the Virus.—The development of drug-resistance to a new chemotherapeutic agent is commonly observed only after the agent has been for some time in clinical use. In the earliest stages of assessing the relative merits of quinoxaline-1:4-dioxide and the antibiotics, we sought to ascertain the ease or otherwise with which such resistance might be engendered. With this object we infected mice and began treatment within a few hours. After a suitable period, determined largely by information concerning the titre of virus at various times in treated animals, we removed the spleens, passed them as a pool to fresh mice, and again started treatment within a few hours. The virus was thus exposed almost continuously to the drug for as long as the experiments lasted. At each passage a few mice were left untreated to demonstrate that virus still persisted in spite of prolonged treatment. Finally, we passed the stock virus in parallel with that exposed to drug to show that repeated transfer had not altered its properties.

Many such experiments were started with various doses of quinoxaline-1:4-dioxide, 7218, procaine-penicillin, or aureomycin. Some ended in failure because after several transfers in the presence of antibiotic the virus under examination failed to appear in the passage-mice, others because it became contaminated with ectromelia. The facts elicited were as follows:

(i) Both psittacosis and lymphogranuloma viruses developed partial resistance in 3 passages and absolute resistance in 4 passages at 14-day intervals to quinoxaline-1:4-dioxide given orally twice daily in a dose of 0.5 mg. At the 4th passage all (30) mice infected with "treated" virus died in spite of therapy, whereas those infected with passaged stock virus responded in the usual manner.

(ii) Similarly psittacosis virus developed considerable resistance to 7218 after 4 to 5 serial passages within 56 to 66 days.

(iii) There was no evidence of resistance to procaine-penicillin (initial dosage 1,000 units once every three days) or aureomycin (initial doses 0.05 to 1 mg.) in 117 or 42 days respectively. The experiments with the latter were particularly troublesome, as virus tended to disappear completely after a few passages; James, Price, and Kneeland (1951) encountered the same difficulty with the virus of feline pneumonitis.

(iv) Virus partially or completely resistant to one of the quinoxaline dioxides responded in the usual manner to aureomycin or procaine-penicillin.

SUMMARY AND CONCLUSIONS

A number of distinct classes of synthetic organic chemicals possess some degree of activity against the largest viruses of the psittacosis-lymphogranuloma group; of these quinoxaline-1:4-dioxide and its substituted derivatives are the most potent we have yet encountered. Some of the substituted derivatives are preferable to the parent compound on the ground of higher antiviral activity coupled with lower toxicity; the best equal aureomycin in activity against lymphogranuloma

venereum in the mouse, but relatively few are as effective against this disease in the chick-embryo or against psittacosis in the mouse. Like aureomycin and unlike penicillin, they are effective whatever the route of inoculation of virus—intraperitoneal, intranasal, or intracerebral. Therapeutic activity has also been noted in man, but in this host toxic side-reactions preclude the use of these compounds.

The compounds do not inactivate virus *in vitro*, but greatly restrict its growth in the mouse even to the point of apparent complete suppression. Nevertheless, as with the antibiotics, after treatment ceases many mice remain carriers of virus indefinitely (more than 277 days), without being contagious for normal animals. Therapeutic activity is not abolished by simultaneous exhibition of synthetic vitamin K. After several serial passages in the presence of the drug the viruses under consideration become drug-resistant.

The therapeutic activity of penicillin is accompanied by considerable morphological changes in the virus, which for much of its period of development continues to grow into large plaques (up to 6 μ in diameter) without subdivision into elementary bodies. Similar giant forms are not seen with the quinoxaline dioxides or aureomycin ; although at one stage plaques rather larger than normal develop, they ultimately divide into elementary bodies.

The quinoxaline dioxides do not influence infections with the viruses of herpes febrilis, ectromelia, mouse-adapted poliomyelitis, influenza A, equine encephalomyelitis, or louping-ill. One of them (7218) was equally devoid of useful activity against *R. burneti* and *Toxoplasma.*

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THE PHARMACOLOGICAL ACTIONS OF A SERIES OF PHENYL ALKANE *p*-ω-BIS(TRIALKYLAMMONIUM) COMPOUNDS*

BY

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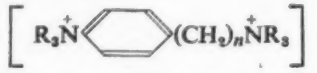
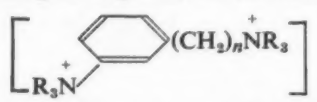
A collaborative programme of research undertaken for the development of a better drug than hexamethonium, which has certain clinical disadvantages, has led to the pharmacological examination of a large number of compounds in several homologous series with a view to obtaining a substance possessing greater duration of action and a more selective effect on ganglia. For the compounds in the present series we are indebted to Dr. J. N. Ashley. They constitute an aromatic series of bis-quaternary compounds,



where $n=0$, 1, 2, 3, 4, 5 or 6, and $R_3=Me_3$, Me_2Et, $MeEt_2$ or Et_3, and of the *meta* isomers,



where $n=1$, 2, 3 or 4, and $R_3=Me_3$. Several compounds were found to have outstanding pharmacological actions, but special attention was paid to one compound in the *para* series where $n=2$ and $R_3=Me_3$ which was examined in more detail; this compound was several times more potent than hexamethonium, and it had a similar type of action. The actions of compounds examined in other series will be described in another paper.

METHODS

The methods employed were those previously described (Wien and Mason, 1951) except that the rate of stimulation of the superior cervical ganglion varied from 12 to 20 instead of 20 per sec. The results shown in Table I were obtained by matching submaximal effects of the test compounds with the standard drugs, hexamethonium bromide and *d*-tubocurarine chloride; each result was the mean value derived from several experiments, but these figures are only approximate because compounds may differ in their slopes of response. Details of other procedures are given in the Results section.

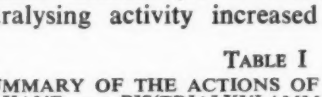
The compounds were all stable substances, soluble in water to give neutral solutions.

RESULTS

A summary of our results is given in Table I. In the *para* series, where $R_3=Me_3$, there was a peak of activity for ganglionic paralysis at the ethane member (M & B 1950); neuromuscular-paralysing activity increased up to the hexane

TABLE I

SUMMARY OF THE ACTIONS OF A SERIES OF PHENYL ALKANE *p*-ω-BIS(TRIALKYLAMMONIUM) COMPOUNDS

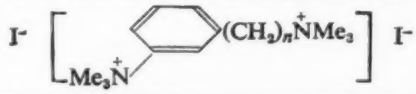


M & B No.	*n*	R_3	Toxicity LD50 mg./kg. i.v. (Mice)	Relative Potencies: Superior Cervical Ganglion (Cat)	Peristaltic Reflex of Ileum (Guinea-pig)	Sciatic-gastrocnemius (Cat)	Phrenic Nerve-diaphragm (Rabbit)
Hexamethonium bromide			50	100	100	—	0·3
d-Tubocurarine chloride			0·2	—	—	100	100
1878	0	Me_3	83	<2	<5	<2	0·1
1899	1	Me_3	34	0·5	<5	<2	0·2
1950	2	Me_3	22	300	250	<2	0·2
2072	2	Me_2Et	9	330	250	<2	0·6
2175	2	$MeEt_2$	36	180	100	7	2·5
2132	2	Et_3	1·8	<2	10*	6	3·0
2034	3	Me_3	20	25	50	<10	0·2
2056	3	Me_2Et	5	50	100	<10	1·0
2278	3	$MeEt_2$	3·2	15	50	—	4·0
2184	3	Et_3	2·2	<4	15*	25	6·0
2071	4	Me_3	1	<5	33*	30*d*	3·0
2082	4	Me_2Et	5	10	25*	5	1·5
2297	4	$MeEt_2$	2·7	5	25*	8	5·0
2272	4	Et_3	1·4	<3	<5*	15	20·0
2268	5	Me_3	1·7	<5	<2	50	6·0
2363	5	Et_3	1·5	<5	<2	50	10·0
2357	6	Me_3	3·1	20*a*	100*b*	100*e*	5·0
2362	6	Et_3	0·6	<12	12·5*c*	100*f*	25·0

* Stimulant effect. *a* Influence of neuromuscular block. *b* Eserine-like effect on rabbit ileum. *c* Acetylcholine-like effect on rabbit ileum. *d* In small doses potentiates the muscle twitch. *e* Not reversed by eserine. *f* Reversed by eserine.

*The results recorded in this paper were communicated to the British Pharmacological Society at Edinburgh in July, 1952.

TABLE II
SUMMARY OF THE ACTIONS OF THE *META* COMPOUNDS



M & B No.	*n*	Toxicity LD50 mg./kg. i.v. (Mice)	Relative Potencies: Superior Cervical Ganglion (Cat)	Peristaltic Reflex of Ileum (Guinea-pig)	Sciatic-gastrocnemius (Cat)	Phrenic Nerve-diaphragm (Rabbit)
Hexamethonium bromide		50	100	100	—	0·3
d-Tubocurarine chloride		0·2	—	—	100	100
2030	1	6	<5	<5	<10	0·1
2064	2	20	150	125	<10	0·2
2090	3	12	100	50	<10	0·3
2139	4	2	100	100	<10	0·5

member (M & B 2357), which was as far as the series was examined. The influence of chain length was illustrated in a striking manner in the sharp increase in ganglion-blocking activity (600 times) in passing from the methane member (M & B 1899) to the next higher homologue. For this latter compound the potency was much the same on both sympathetic and parasympathetic ganglia, but we would emphasize that these results were based on only two types of ganglia in two different species (the superior cervical ganglion of the cat, and the ganglia mediating the peristaltic reflex of the guinea-pig intestine).

The results in Table I and Fig. 9 (see below) bring out the interdependence between activity and chain length in the *para* series, which followed a similar pattern to that in the methonium series (Paton and Zaimis, 1951). The results for the four compounds of the *meta* series are given in Table II. Since the ethane member (M & B 2064) was only one half as active as its *para* isomer (M & B 1950) the *meta* series was not examined in greater detail.

Just as replacement of methyl by ethyl groups in the methonium series exerts a profound effect on the intensity and type of action of various members (Wien, Mason, Edge, and Langston, 1952), so in this series the complete substitution of methyl by ethyl groups caused generally an increase in toxicity, a reduction or complete suppression of ganglion-blocking activity, and an increase in neuromuscular-blocking properties.

Of particular interest were the two hexane members which were both as active as *d*-tubocurarine in producing neuromuscular paralysis in the cat under chloralose. But one (M & B 2357, where $R_3=Me_3$ and $n=6$) had an action similar to that of decamethonium, whereas the other (M & B 2362, where $R_3=Et_3$ and $n=6$) had an action like *d*-tubocurarine in the cat. This is in sharp contrast to the complete replacement of methyl by ethyl groups in decamethonium, which considerably reduces activity (Paton and Zaimis, 1952).

Actions of M & B 1950 : Phenyl Ethane p-*ω*-*bis(trimethylammonium Iodide)*

Paralysis of Autonomic Ganglia.—On the superior cervical ganglion of the cat M & B 1950 was three times as potent as hexamethonium bromide, and in equipotent doses the duration of action was somewhat longer (Fig. 1).

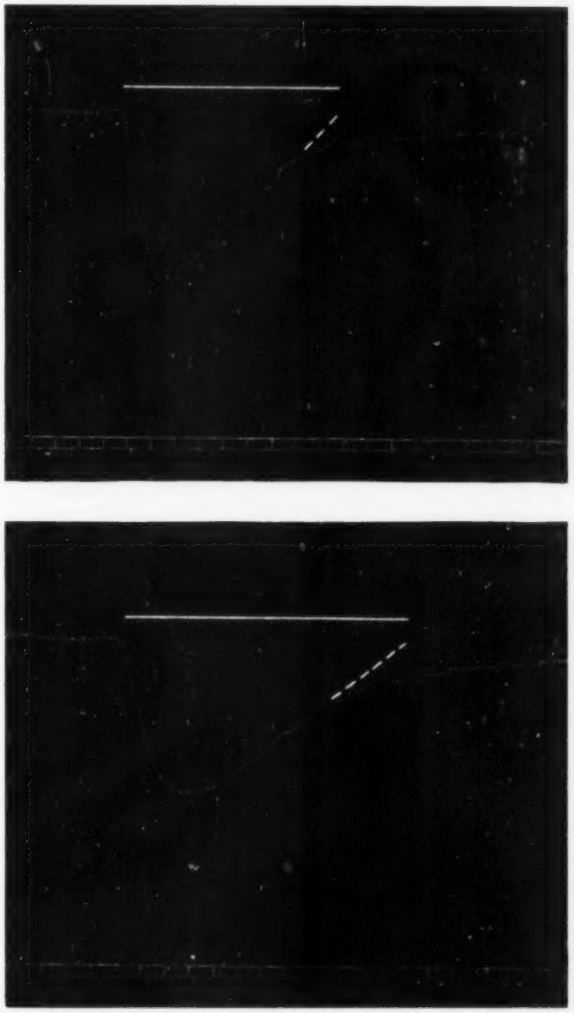


FIG. 1.—Cat, chloralose. Record of contractions of nictitating membrane on sustained preganglionic excitation of cervical sympathetic at 12 stimuli per sec. Upper record shows effect of 0.8 mg. intravenously of hexamethonium bromide, and lower record of 0.25 mg. intravenously of M & B 1950. (Time= 30 sec.) Note slightly prolonged duration of latter compound.

The onset of action was similar, though slightly slower and more gradual in reaching a maximum ; the recovery phase was correspondingly slower. The doses required for these effects on the nictitating membrane (ranging from 0.05 to 0.4 mg. depending on the rate of stimulation) had little depressor effect on the arterial blood pressure. When the rate of stimulation was decreased from 20 to 5 per sec. the sensitivity of the ganglion to paralysis by both the phenethyl compound and hexamethonium was decreased considerably, about five times, the ratio of their potencies remaining the same. The stimulant action of nicotine on the blood pressure of the dog under chloralose was inhibited, and the intensity and duration of the pressor effect of adrenaline were slightly enhanced (Fig. 2).

The compound was two and a half times as potent as hexamethonium in preventing the peristaltic reflex of the guinea-pig intestine. The preparation readily differentiated between two-fold increases in the dose, and doses of 150 to 300 μg. in a 50 ml. bath caused partial to complete inhibition of the reflex ; recovery occurred quickly after washing out once or twice.

The slowing of the pulse and the fall in blood pressure in the cat on vagal excitation were abolished with doses of 0.25 mg./kg., but the depressor action of acetylcholine was unmodified. It was difficult to obtain reliable comparative figures by this method because of the long duration of the effects, sometimes for several hours.

The mydriatic effect in the mouse, due to paralysis of the ciliary ganglion, was four times greater than that of hexamethonium. On intraperitoneal injection, after 10 minutes, the potency figure was assayed as 4.19, with fiducial limits of 3.5 to 5.1 for P=0.95 (experiment by Mr. N. D. Edge).

Mode of Action.—In several ways we have shown that the compound M & B 1950 has a mode of action similar to that of hexamethonium. The stimulant effect of nicotine on the intestine was abolished, leaving unaltered the effects of acetylcholine, histamine, and of pilocarpine, exactly similar to a result previously described for a homologue of hexamethonium (Wien and Mason, 1951). Freedom from an atropine-like action was confirmed in the cat by the absence of any inhibitory effect with an intravenous dose of 2 mg. on salivary flow induced by carbamylcholine, compared with the effect of 5 μg. atropine which markedly inhibited the flow. The paralysis of transmission occurred most probably at the gang-

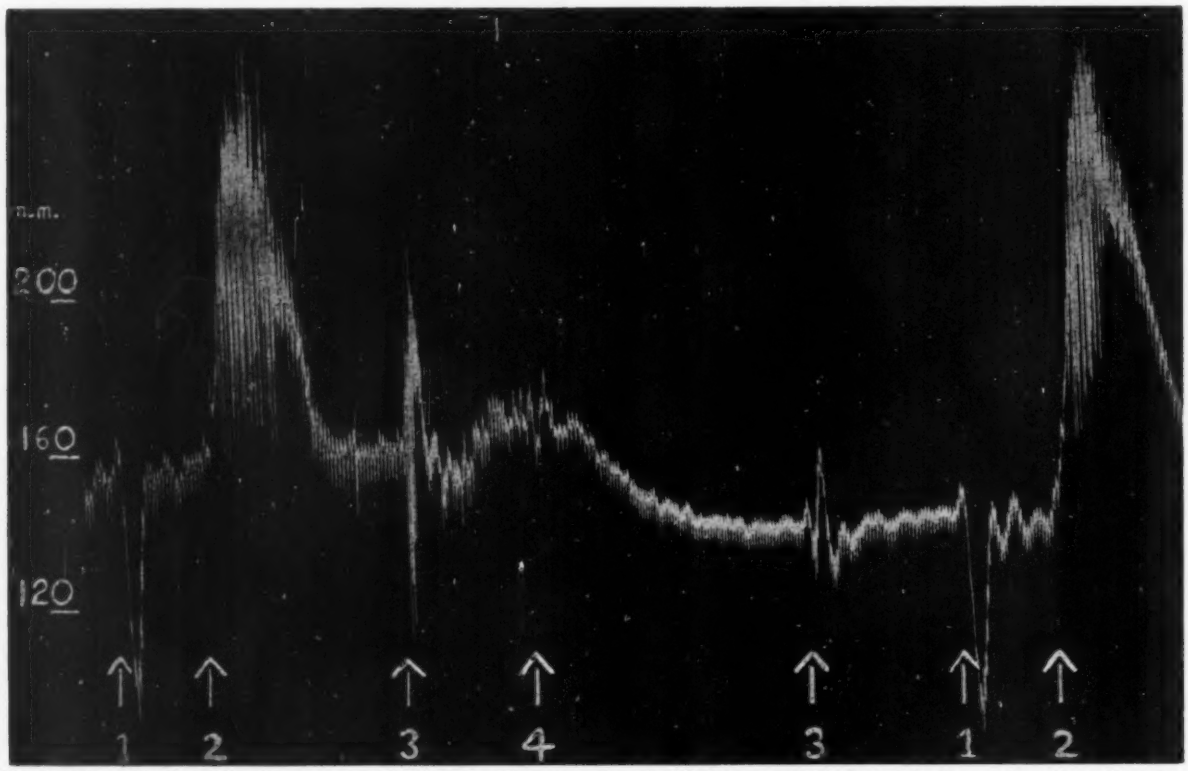


FIG. 2.—Dog, chloralose. Blood-pressure record. Injections intravenously. At (1), 50 μg. acetylcholine; (2), 50 μg. adrenaline; (3), 0.5 mg. nicotine acid tartrate; (4), 0.05 mg./kg. M & B 1950.

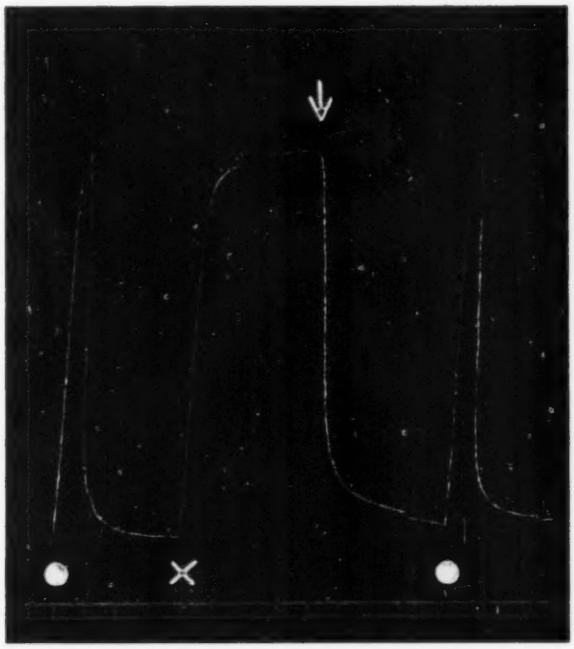


FIG. 3.—Cat, chloralose. Contractions of nictitating membrane. At dots, postganglionic stimulation of cervical sympathetic for 15 sec.; at cross, sustained preganglionic stimulation. At arrow, intravenous injection of 0.5 mg. M & B 1950. (Time = 30 sec.)

lion synapse, since the block was confined to preganglionic, not extending to postganglionic, excitation of the cervical sympathetic (Fig. 3); furthermore, the nictitating membrane reacted normally to adrenaline during a complete paralysis.

Though it has not been proven that acetylcholine is normally released during paralysis of the superior cervical ganglion or that the ganglion is not depolarized, the preceding evidence taken collectively strongly suggests a competitive type of action like hexamethonium. And in all experiments no actions have been encountered which could be explained in any other way. There was no evidence of any anticholinesterase activity, determined manometrically, at a concentration as great as 3×10^{-3} M.

Toxicity

(i) *Acute Toxicity*

Mice.—The intravenous, subcutaneous, and oral LD50 figures were 22 ± 2.3, 83 ± 8.5, and 575 ± 73 mg./kg. respectively, the ratios being 1:4:26. These figures show this compound to be about two and a half times as toxic as hexamethonium bromide. Death resulted from respiratory failure.

Rabbits.—Each dose was tested by injection into six adult rabbits and the injections were made into the marginal ear vein. Doses up to 3 mg./kg. caused flushing of the ears, but no other obvious symptoms. Much larger doses (30 mg./kg.) caused depression of the respiratory rate and head-drop, typical of neuromuscular paralysis; a dose of 40 mg./kg. was fatal.

Cats.—Injections were made into the saphena vein, three cats being used for each dose. Doses of 0.5 to 2.0 mg./kg. of M & B 1950 caused immediate dilatation of the pupil, the heart rate was slowed, and in some animals there was ataxia. Recovery occurred within thirty minutes. A dose of 10 mg./kg. was fatal to two of three cats, owing to respiratory failure; the heart continued to beat strongly for several minutes after respiration had ceased.

(ii) *Chronic Toxicity*

Rats.—Daily subcutaneous injections to young albino rats (Wistar strain) caused no retardation in the growth rate over a period of two weeks. Six rats, 40 to 50 g. body weight, were in each group, and the treated animals received a daily dose of 16 mg./kg., which is far in excess of the pharmacologically effective dose. Litter-mate controls showed a mean increase in weight of 16 ± 1.6 g. compared with 15 ± 2.6 g. for the treated group.

Rabbits.—During daily intravenous injections of 3 mg./kg. to four rabbits for a period of one month no ill effects were observed in the health of the animals, as shown by urine analysis (absence of glucose, protein, and blood) and blood analysis (normal values for glucose and urea). Flushing of the ears was observed, but no local damage was caused to the veins. Dr. R. Williamson, of the Department of Pathology, Cambridge, has kindly allowed us to quote from his report on the examination of tissues from these animals after they were killed. "In each animal kidney, liver, heart muscle, spleen, bone marrow, and lungs were examined. In some animals the spinal cord, pancreas, portion of intestine, suprarenal and lymph nodes were examined. In all animals the condition of blood vessels, nerves, or other common structures was noted. In one rabbit in the kidney section there was a small area of inflammation due to an ascending pyelitis. In the heart there was evidence of slight scarring, probably due to intracardiac puncture (which had been carried out to obtain samples of blood). In the trachea there was some infiltration of the mucous membrane with polymorphs; a tracheitis. No other pathological changes were found in any of the sections, and there is nothing to suggest that the compound was responsible for these changes."

Guinea-pigs.—Each animal in a group of six guinea-pigs received a daily subcutaneous injection of 8 mg./kg. for one month. During this period, and for another three weeks after stopping the injections, a complete blood-picture examination was made twice weekly of each animal. No abnormalities were observed except a slight increase in large lymphocytes which fell to normal when treatment was discontinued (we are indebted to Mr. W. A. Freeman for the results of this experiment).

Cardiovascular System

Blood Pressure.—In the dog under chloralose the fall of arterial blood pressure after an intravenous injection of 0.05 to 0.25 mg./kg. (see Fig. 2) depended not only on the size of the dose but on the initial level of the blood pressure. The fall appeared slowly and reached a maximum within a few minutes. Its duration depended on the dose and often there was not complete recovery to the initial level. This effect, resulting most probably from the release of sympathetic tone in the vessels, is characteristic of ganglion-blocking drugs, and once the tone in the vessels has been released no further fall can be produced. Though the first few doses produced a fall, subsequent injections had little effect even after several hours. There was hardly any effect on the respiration in the cat (chloralose) with an intravenous dose of 5 mg./kg., which produced a fall in blood pressure of 65 mm.; 10 mg./kg. depressed the respiration but did not completely inhibit it.

Heart.—No direct action was observed on the perfused heart of the rabbit or cat (Langendorff's preparation). In the dog the effect was variable, but a decrease in coronary flow was never observed. Two experiments typify our findings; in one the coronary outflow was increased from 74 to 90 ml./min. with 20 μg. of adrenaline, and from 84 to 120 ml./min. with 0.5 mg./kg. intravenously of M & B 1950; in the other, though adrenaline in the same dose increased the flow from 76 to 100 ml./min., 0.5 or 1.0 mg./kg. of the compound left the flow unaltered.

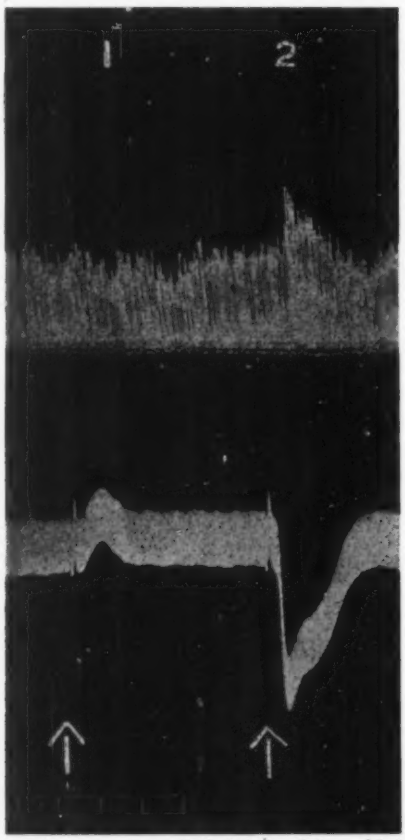


FIG. 4.—Perfusion of vessels of hind-limb of dog with heparinized blood. Upper record, venous outflow; lower record, arterial pressure. Intra-arterial injections of (1) 3 mg. M & B 1950; (2) 3 mg. hexamethonium bromide. (Time=30 sec.)

Vessels.—The vessels of the hind-limb of the dog (severed from the trunk) were perfused by means of a Dale-Schuster pump with heparinized blood which was oxygenated through the lungs. In several experiments in which the compound was injected into the arterial supply in a dose of 3 mg. no effect on the vessels was demonstrable, though hexamethonium in the same dose caused a perceptible vasodilatation (Fig. 4).

Gastric Secretion.—In the dog under chloralose in which a secretion of acid gastric juice is promoted by vagal excitation, as previously described, the intravenous injection of M & B 1950 was very effective in reducing the volume, acidity and, to a lesser extent, the peptic activity of the juice. Seven experiments were performed and the results are summarized in Fig. 5. The dose range was from 0.05 to 0.40 mg./kg.; the highest dose caused achlorhydria for at least three hours, the maximum period of observation. On intravenous injection of the compound, when a steady state of acid secretion was established, the inhibitory effect reached a maximum within about 30 minutes, and it was noticeable that inhibition of gastric secretion outlasted the vagal blocking action on the heart. When the compound was given via the small intestine (introduced by catheter through the abdominal incision), in a dose of 1 to 5 mg./kg., the effect was slower in starting, but achlorhydria was obtained, indicating that it is absorbed from the intestine.

Other Actions.—The knee-jerk reflex in the cat (chloralose) was unmodified with doses as large as 10 mg./kg. intravenously. There was no evidence of the release of histamine, since after atropine and nicotine there was no effect on the blood pressure in a dose of 5 mg./kg. Injection into the cisterna magna in the conscious rabbit produced slight depression but no convulsive movements. The compound had no anticholinesterase activity even in a concentration of 1,000 μg./ml.

Urinary Excretion in Rabbits.—Estimations were made by the reineckate method of Zaimis (1950), and groups of four rabbits were used to

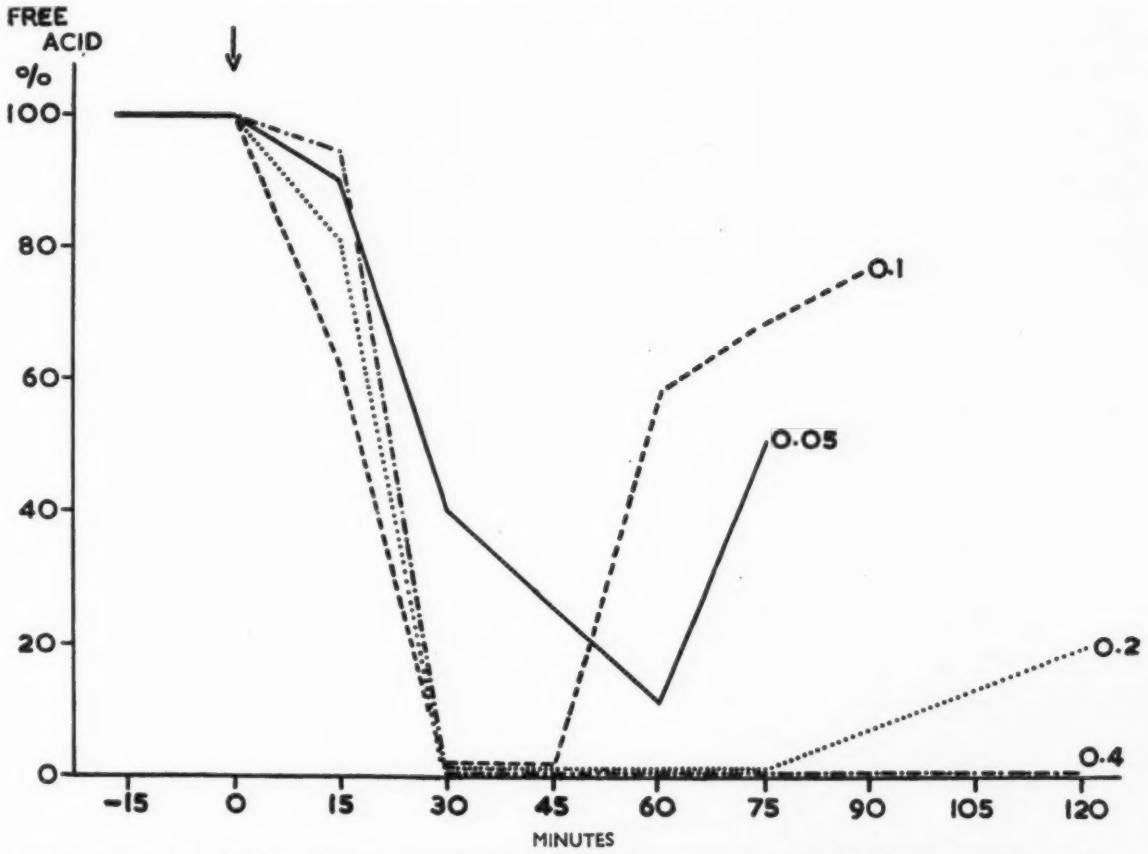


FIG. 5.—The effect of M & B 1950 on gastric secretion in dogs, induced by vagal stimulation (Babkin's method as used by Wien and Mason, 1951). Curves showing the effects of varying doses, 0.05 to 0.4 mg./kg. intravenously, on the secretion of free acid (ml. 0.1N-HCl), expressed as a percentage of the pre-injection level (ordinate) against time in minutes (abscissa). The drug was not injected until a steady level had been attained for at least 1 hour, although only a 15-minute control period is shown.

test each dose. After a single intravenous injection of 15 mg./kg., from 68% to 89% of the dose was excreted within 24 hours. From 54% to 86% was found in the urine within 48 hours after 20 mg./kg. subcutaneously; from 55% to 76% after 20 mg./kg. intramuscularly; but only 9% to 21% was recovered in the urine within five days after 300 mg./kg. given by stomach tube.

Neuromuscular Paralysis (Para Series of Compounds)

Influence of Length of Alkane Chain

(i) *Where* $R_3=Me_3$.—Neuromuscular-blocking properties first appeared in the butane member (one-third as active as *d*-tubocurarine), and increased to the hexane member which was approximately as active, superficially, as *d*-tubocurarine in the sciatic-gastrocnemius preparation of the cat (Table I). Though injections were spaced sufficiently far apart, the effect of one compound might have influenced the other and further study, using other species and preparations, is required to obtain a more precise evaluation.

(ii) *Where* $R_3=Et_3$.—Activity first appeared in the ethane member (6% of *d*-tubocurarine), and increased to the hexane member, which was also as active as *d*-tubocurarine in the cat. Higher homologues have not as yet been examined to see whether the peak of activity occurs beyond the hexane members.

Results on the phrenic nerve-diaphragm preparation showed the same general trend of events in relative activities, though the absolute values were lower, which may be due to species or muscle differences, or to some other factor. The two hexane members were exceptional; they were equally active in the cat, but the bis-Et_3 compound was five times as active as the bis-Me_3 compound by the phrenic nerve-diaphragm method.

Type of Action.—The well-known distinction between depolarizing and competitive block at the neuromuscular junction can be readily tested, on

the frog rectus muscle, by intravenous injection in the chick, by the ability of neostigmine to reverse the paralysis, and by the response to a tetanus; these methods have been applied to the present compounds.

The compounds with $R_3=Me_3$, and with $R_3=Et_3$, had different modes of action. When $R_3=Me_3$, the pentane and hexane members produced contractions of the frog's rectus, which were inhibited by *d*-tubocurarine; and subthreshold concentrations potentiated the action of acetylcholine. In the chick these compounds produced

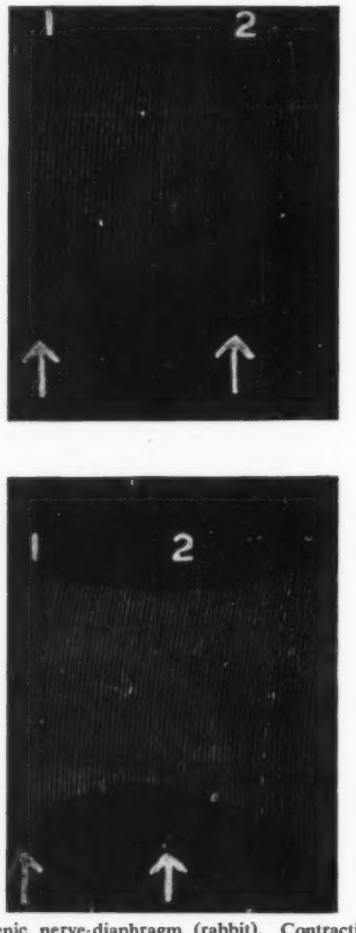


FIG. 6.—Phrenic nerve-diaphragm (rabbit). Contractions of diaphragm on stimulation of nerve (6 stimuli per min.). Upper record, (1) 1.5 mg. M & B 2357 and (2) 125 μg. neostigmine. Lower record, (1) 0.4 mg. M & B 2362 and (2) 150 μg. neostigmine.

a spastic paralysis in intravenous doses of 0.25 to 1.0 μg./g. compared with 1.0 to 5.0 μg./g. for decamethonium iodide. Neuromuscular block of the phrenic nerve-diaphragm preparation by the hexane member was not reversed by neostigmine (Fig. 6). A tetanus of cat's gastrocnemius muscle, partially paralysed by the hexane member, was well sustained (Fig. 7).

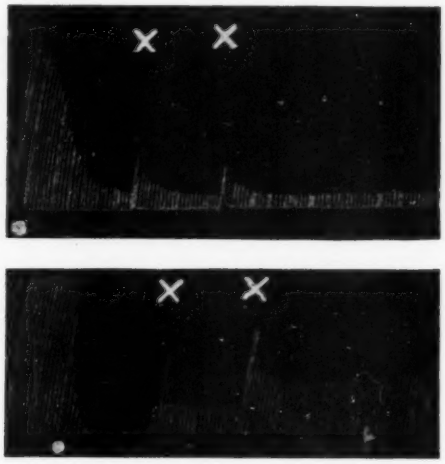


FIG. 7.—Cat, chloralose. Gastrocnemius twitch tensions on stimulation of sciatic nerve (12 stimuli per min., 0.5 msec. duration). Upper tracing, 0.5 mg. M & B 2357 intravenously at dot; at crosses, tetanus of 50 stimuli per sec. for 2 to 5 sec. Lower tracing, 0.5 mg. M & B 2362 intravenously at dot; at crosses, tetanus as before.

With the $R_3=Et_3$ compounds, the pentane and hexane members did not produce contracture of the frog rectus, but they did block the response to acetylcholine. Intravenous injections (0.5 to 1.5 μg./g.) in the chick produced a flaccid paralysis comparable with that of *d*-tubocurarine chloride (0.5 μg./g.). On the phrenic nerve-diaphragm preparation, neuromuscular block produced by the hexane member was reversed by neostigmine; in the cat (under chloralose) a tetanus of the gastrocnemius muscle, almost completely blocked by this compound, was not well sustained, and after the tetanus there was a partial reversal of the block.

The two hexane compounds consequently had contrasting actions: the compound in which $R_3=Me_3$ resembles decamethonium, and the compound in which $R_3=Et_3$ resembles *d*-tubocurarine. This was borne out by the observation that the bis-Me_3 compound was able to reverse the paralysis produced by the bis-Et_3 compound (Fig. 8), just as decamethonium can reverse a *d*-tubocurarine paralysis. The paralysis caused by the bis-Me_3 compound was also reversed by the ethane member, $R_3=Me_3$, just as a decamethonium paralysis is reversed by hexamethonium.

DISCUSSION

In the present aromatic series of bis-quaternary compounds there were several members of outstanding pharmacological interest. M & B 1950, phenyl ethane *p*-ω-bis(trimethylammonium iodide),

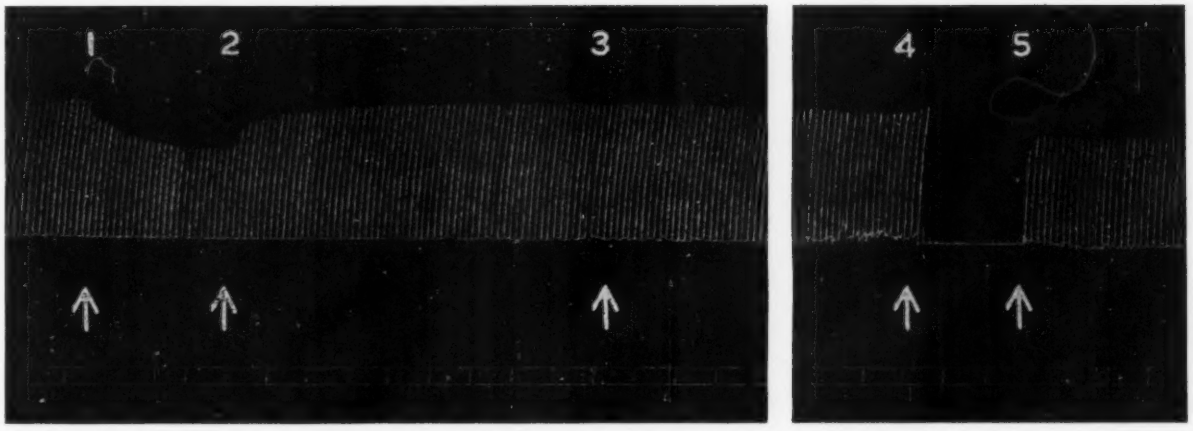


FIG. 8.—Cat, chloralose; same preparation as Fig. 7. Intravenous injections of (1) 0.75 mg. M & B 2362 which caused a partial block, reversed by (2) 0.5 mg. M & B 2357. A subsequent injection (3) of 1.0 mg. M & B 2362 was ineffective. Later, (4) 5 mg. of M & B 2362 caused complete paralysis which was immediately reversed by (5) 5 mg. of M & B 2357. (Time = 30 sec.)

was three times as potent as, and slightly longer in its duration than, hexamethonium on the cat's superior cervical ganglion, with a similar type of action in specifically paralysing transmission at the ganglion synapse. It did not possess any greater selectivity on the ganglia studied. The compound M & B 2072 was slightly more active, but the difference was not sufficiently great to warrant further study.

Two higher members, phenyl hexane *p*-ω-bis-(trimethylammonium iodide), M & B 2357, and the bis-triethyl analogue, M & B 2362, possessed superficially about the same activity as *d*-tubocurarine in the cat under chloralose. But their types of action were quite different; the actions of the former compound resembled a block by depolarization, whereas those of the latter were like a competitive block. These two compounds have not been studied in the same detail as M & B 1950, but the compound M & B 2362 in particular offers promise as a useful neuromuscular-blocking drug.

By ascending the series and increasing the inter-quaternary distance it was found that there were two active compounds of similar neuromuscular-blocking potency, but of different types of action. In this way this aromatic series differed from the methonium series.

The sharp definition of the peak of ganglion-blocking activity (Fig. 9) can be considered of some significance. This might be due to the aromatic nucleus conferring greater rigidity in the molecule, thus reducing the number of possible configurations which the molecule can take up in which a fit with the receptor groups is not obtained. From measurements on molecular models the compound M & B 1950 has a chain length of 8.5 Å between the polar groups in the fully extended state, intermediate between that of pentamethonium and hexamethonium, indicating possibly a better fit on the receptors. But we would agree with the very apt comment of Paton and Zaimis (1952) that "the literature is full of shipwrecks in the quicksands of 'structure-action-relationship,' and it is obvious that our biophysical knowledge is inadequate to the pharmacological strains placed on it."

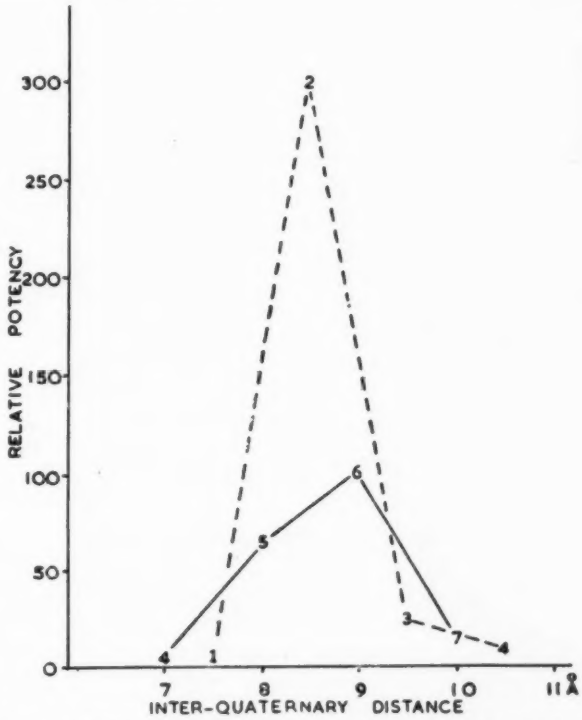


FIG. 9.—Graph relating potency (paralysis of superior cervical ganglion) and interquaternary distance in Å units in two homologous series. Solid line, $(CH_3)_3N^+(CH_2)_nN^+(CH_3)_3$. Broken line, $(CH_3)_3N^+C_6H_4(CH_2)_nN^+(CH_3)_3$. Figures in the curves show the values for *n*.

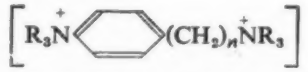
If ganglion-blocking activity depended simply on the distance separating the two polar groups, then the propane member of the *meta* series and the ethane compound of the *para* series, with equivalent interquaternary distances, might be expected to be equipotent. That this was not so demonstrates the fallacy of this supposition, even when two isomers are compared. The successive replacement of methyl by ethyl groups on both nitrogen atoms did generally produce throughout the series, by previous analogy (Wien *et al.*, 1952), a decrease in ganglion-blocking activity and an increase in neuromuscular-blocking properties.

The contrasting actions of the two hexane compounds, M & B 2357 and M & B 2362, have been referred to, and their pharmacological interest and potential value as neuromuscular-blocking substances invite more detailed investigation. It is intriguing that the transition from one type of action to another, in the same species (cat), is brought about by a relatively small change in chemical structure. It is also interesting that the injection of one compound should reverse the effect of the other (Fig. 8).

The relative activities on the superior cervical ganglion and on the peristaltic reflex of the intestine were compared, and one member, M & B 2175, where $n=2$ and $R_3=MeEt_2$, had a more selective effect on sympathetic ganglia. But, apart from the appearance of mixed actions in this compound, such a comparison was likely to be misleading, because of the species differences and the totally different types of preparation employed (whole animal and isolated tissue). A far better comparison would have been the simultaneous examination on different ganglia in the same animal, and experiments in this direction have been performed on the ciliary and pelvic ganglia and chorda tympani in the cat, the results of which will form the subject of another communication.

Summary

1. The series of phenyl alkane *p*-ω-bis(trimethylammonium) salts,



showed a sharp peak of activity for ganglionic paralysis where $R_3=Me_3$ at $n=2$. As far as the series was examined maximal neuromuscular-blocking activity was observed at $n=6$. This roughly corresponded to the two peaks in the methonium series (on the assumption that the phenyl group is equivalent to 3 to 4 methylene groups). These maxima occurred at chain lengths of approximately 8.5 Å and 12.5 Å in the fully extended state.

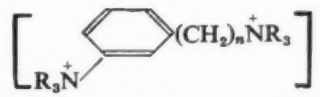
2. The ethane compound ($R_3=Me_3$) was three times as potent as hexamethonium (with a slightly longer duration of action). Two hexane compounds ($R_3=Me_3$ and Et_3 respectively) were approximately as active as *d*-tubocurarine in the cat (sciatic-gastrocnemius preparation).

3. The influence of chain length in this series was illustrated by the remarkably sharp peak of ganglion-blocking activity, since there was a 600-fold increase in passing from the methane to the ethane member.

4. In all the experiments performed the properties of the ethane compound were essentially similar to those of hexamethonium.

5. The complete substitution of methyl by ethyl groups at $n=6$ led to an alteration in the type of neuromuscular-block from one which resembled decamethonium to one like *d*-tubocurarine, without loss of activity. But the modes of action of these two compounds have not been investigated in detail. The neuromuscular paralysis in the cat caused by the hexane compound, where $R_3=Me_3$, was reversed by the ethane compound, where $R_3=Me_3$; the former compound also reversed the block due to the hexane compound, where $R_3=Et_3$.

6. Only four compounds were examined in the *meta* series,



The second member was only one half as active as its corresponding isomer in the *para* series.

We are indebted to Dr. J. N. Ashley, who is describing the chemical work elsewhere, for the compounds, and to Dr. B. J. Heywood for the measurements on molecular models. We wish to thank Mrs. E. Zwartouw, Mr. G. T. Langston, and Mr. N. D. Edge for much assistance, and the directors of May & Baker Ltd. for permission to publish these results.

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Brit. J. Pharmacol. (1953), **8**, 315.

THE PART PLAYED BY BRONCHIAL MUSCLES IN PULMONARY REFLEXES

BY

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A number of reflexes have been described whose receptors are in the lungs and which, on the efferent side, alter the rate and depth of breathing. It would be reasonable to suppose that some of these reflexes might also alter the tone of the bronchial muscles. Gilding and Nutt (1944) showed that stored plasma not only caused an alteration of respiratory movements which was abolished by cutting the vagi, but also caused large increases in intrapleural pressure, which they ascribed to bronchoconstriction. However, the point has not been thoroughly investigated, and it was therefore thought worth while studying the changes of tidal air in cats after injection of 2-α-naphthyl ethyl isothiourea, diphenhydramine, and veratrine, all of which substances excite pulmonary reflexes which alter breathing (Dawes, Mott, and Widdicombe, 1951; Jones, 1952).

The methods in common use for measuring tidal air do not permit direct measurement of changes in bronchial tone in the whole animal. They do not distinguish between bronchoconstriction and a displacement of air due to an increase of the volume of blood in the lungs. Experiments have therefore been performed to show the nature and magnitude of the effects on ventilation of changes in the pulmonary blood volume.

METHODS

Cats were anaesthetized with chloralose (50 or 60 mg./kg.), the chest was opened widely, and their lungs were ventilated with a pump delivering a constant stroke volume. The tidal air was measured by an "overflow" method (Konzett and Rossler, 1940), in which a fixed volume of air entering the trachea was allowed to rise to a predetermined pressure, and the excess of air escaping from the system was measured at atmospheric pressure. The volume measured varies inversely with the tidal air and for qualitative purposes is a sufficient indication of changes in tidal air, but it is not quantitatively accurate, since the air actually enters the lungs at positive pressure. Further errors are introduced by the fact that equilibrium is not reached in the bronchial system during the respiratory cycle. The system was calibrated directly by placing the excised lungs or a rubber bag in a chamber connected to a second float recorder (F_2, Fig. 1). It was found that, for a constant pump stroke and intratracheal pressure, the distribution of air between "lungs" (F_2) and overflow recorder (F_1) (which could be altered by varying the size of the inlet to the lungs or overflow

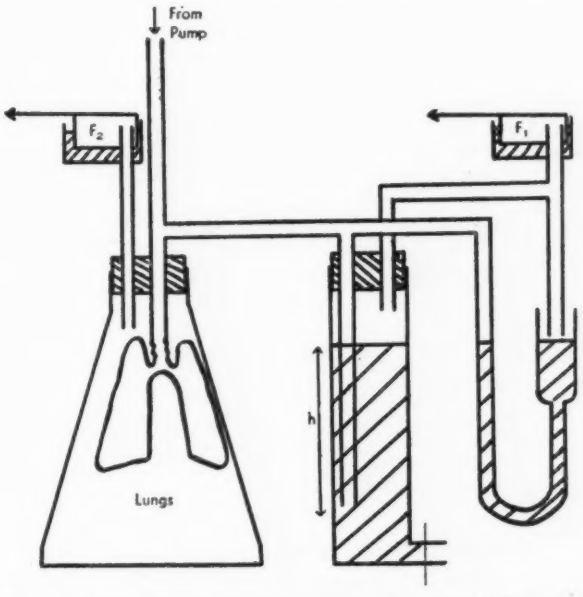

FIG. 1.—Overflow apparatus for measuring tidal air; h represents pressure at which lungs are ventilated.

with screw-clips) showed a linear relation. Hence the true change in lung volume (F_2) at each pump stroke, corresponding to the observed volume (F_1), could be calculated. The relation between F_1 and F_2 was always linear, but the slope varied with the elasticity of the bag, so that a bag had to be chosen which behaved similarly to the lungs *in vivo*; after excision the elasticity of the lungs was found to be greatly changed.

Pulmonary arterial pressure was measured with a condenser manometer using the method described by Dawes, Mott, and Widdicombe (1951). Left atrial

pressure was recorded either with a condenser manometer or with a saline manometer equipped with an overflow device to permit bleeding.

Drugs used were: 2-α-naphthyl ethyl isothiourea; diphenhydramine hydrochloride (Parke Davis); veratrine BPC (B.D.H.); and chlorbismol (May and Baker).

Results

2-α-Naphthyl Ethyl Isothiourea.—The action of 2-α-naphthyl ethyl isothiourea was studied in 57 cats. In addition to the slowing of the heart, fall in blood pressure, and reflex changes in natural breathing already described (Dawes and Fastier, 1950; Dawes, Mott, and Widdicombe, 1951), this compound caused a decrease in tidal air of 3–20 ml. (5–16%) in all healthy animals (Fig. 2). This

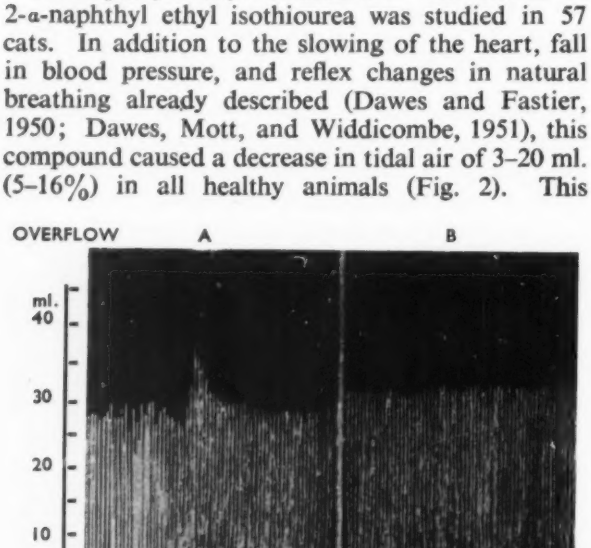

Fig. 2.—Cat, 3.8 kg.; chloralose. Records (above) of overflow from artificial respiration apparatus (inversely related to tidal air), and (below) of blood pressure. Time marks, 10 sec. intervals. A and B, intravenous injection of 50 μg. 2-α-naphthyl ethyl isothiourea. Between A and B cervical vago-sympathetic trunks cut. The tidal air is reduced by the injection when the vagi are intact.

effect was not obtained if there was the slightest respiratory infection (even though the depressor effect was present), and it was subject to rapid tachyphylaxis. The first response was often much larger than those which followed, and there was no correlation between the size of the respiratory response and the depth of the fall of blood pressure. After repeated doses it was usual to find that the reduction in tidal air ceased, while the depressor reflex persisted, although occasionally the opposite was observed. Unlike the effect on blood pressure the magnitude of the tidal air response seemed to bear no relation to the dose given (10–200 μg.). These points support the contention of Dawes and Mott (1950) and Dawes, Mott, and Widdicombe (1951) that the respiratory and depressor reflexes caused by the amidines are distinct. In nine cats in which both vagus and sympathetic were cut in the neck the effect of 2-α-naphthyl ethyl isothiourea on the tidal air was abolished (Fig. 2); in a tenth cat the effect was obtained after vagotomy, but this was abolished when all further nerve fibres running alongside the carotid arteries were cut. In six cats first the vagi and then the sympathetic were cut. In four of these the reduction of tidal air was abolished after vagal section only; in the other two the effect was still present until the sympathetics were also cut. It is therefore possible that there is some crossing of fibres between the main vagus and cervical sympathetic trunks.

In four cats in which 2-α-naphthyl ethyl isothiourea caused a large reduction of tidal air, this was diminished but not abolished by atropine, 2–5 mg. (Table I). In one cat a small response

Table I

Experiment No.	Dose of 2-α-Naphthyl Ethyl Isothiourea	Decrease in Tidal Air (ml. at Atmospheric Pressure)
1	50 μg.	12
	50 ,, +compensator	19·5
2	50 ,,	7
	50 ,, +compensator	12
3	50 ,,	7
	50 ,, after atropine	2·5
4	75 ,,	7
	75 ,, after atropine	3
5	25 ,, intravenously	10
	25 ,, to left atrium	1·3
	25 ,, intravenously	10·5
6	30 ml. saline intravenously	11·5

was not reduced, and in two other cats small responses were abolished.

In three cats repeated injections of 2-α-naphthyl ethyl isothiourea into the left atrium had no effect on the tidal air, while injections into the right atrium, intravenously, or into the pulmonary artery were effective (Table I). The receptors for the reflex effect on tidal air are therefore probably in the lungs.

In order to establish the independence of the reduction in tidal air and the fall of blood pressure, a blood pressure stabilizer was inserted into the

abdominal aorta in seven cats. In the presence of a functioning stabilizer there was an even greater reduction of tidal air after the administration of 2-α-naphthyl ethyl isothiourea than in controls (Table I). Two possible explanations suggested themselves. First, the normal decrease in tidal air could be a balance between a reflex decrease in tidal air due to 2-α-naphthyl ethyl isothiourea, and a reflex increase in tidal air consequent on the fall in blood pressure, such as Daly and Schweitzer (1952) have described in the dog. Secondly, when the stabilizer operates, a large volume of fluid (60–70 ml.) flows into the animal to keep the blood pressure constant; this might increase the pulmonary blood volume and displace air from the lungs. We have no evidence for or against the first hypothesis, but in favour of the second we were able to show that when the stabilizer operated there was a very large rise in left atrial pressure. Moreover, large volumes of saline injected intravenously or into the right atrium (20–40 ml.) produced considerable reductions in tidal air (Table I), even after section of the vagus and sympathetic chains in the neck. The question then arose as to whether the action of 2-α-naphthyl ethyl isothiourea on the tidal air was wholly or partly due to an increase in lung blood volume. Evidence for vascular changes in the lungs was therefore sought.

Measurements of left atrial pressure after administration of 2-α-naphthyl ethyl isothiourea were made in 12 cats with a saline, and in three with a condenser manometer. The mean rise in pressure was 3 cm. blood (limits 0–7 cm.). There was a rise in both systolic and diastolic pressures with gross changes in wave form (Fig. 3). In two cats in which a blood pressure stabilizer was operating the rise in pressure after the isothiourea was 9 and 12 cm. In three cats measurement of pulmonary arterial pressure confirmed the observations of Dawes, Mott, and Widdicombe (1951), and showed an increase in the pulse pressure, the systolic pressure rising and the diastolic pressure falling (Fig. 3). These measurements suggest the presence of pulmonary vascular changes, but not necessarily of changes in lung blood volume. If the decrease in tidal air were due solely to accumulation of blood in the lungs it might be possible to eliminate this effect by preventing the rise in left atrial pressure, although it was observed that there was no correlation between the size of the effect on tidal air and the rise of left atrial pressure. In nine cats an overflow device was inserted into the left auricular appendage; the overflow prevented a rise in pressure and the amount bled varied from 4–20 ml. In every experiment a reduction of tidal air was still obtained, and was little if at all diminished, although quantitative comparisons are difficult because of tachyphylaxis. This procedure might not necessarily prevent a blood volume change in the lungs, but it should reduce it.

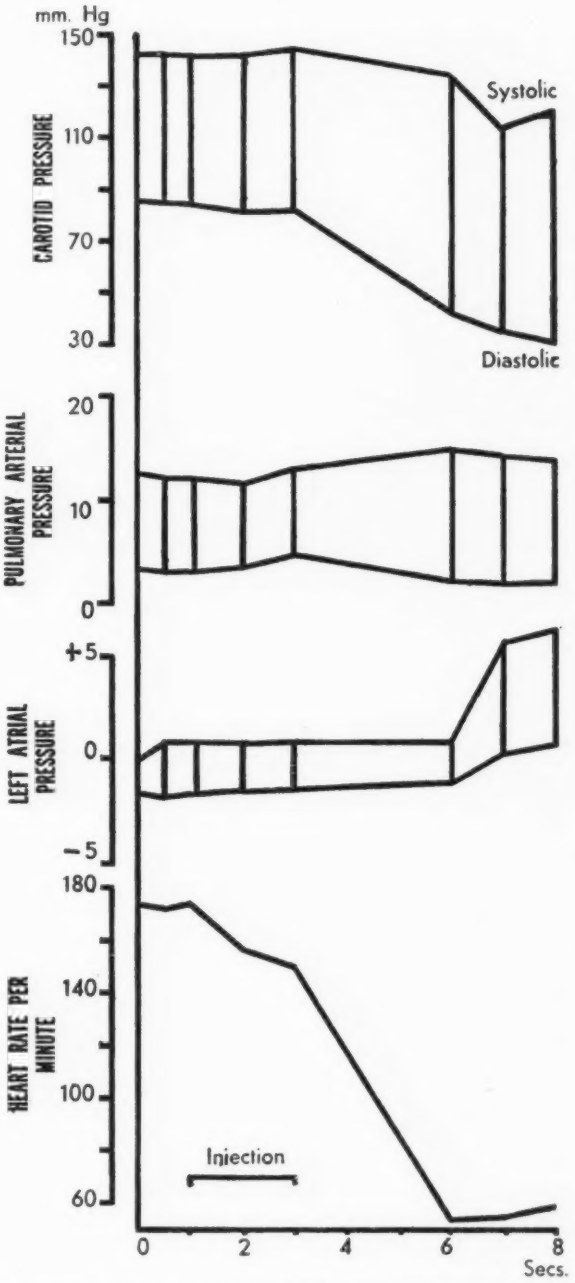

FIG. 3.—Cat, kg.; chloralose. Effect on circulation of injecting 100 μg. 2-α-naphthyl ethyl isothiourea (I.V.). Pressure measurements with condenser manometers.

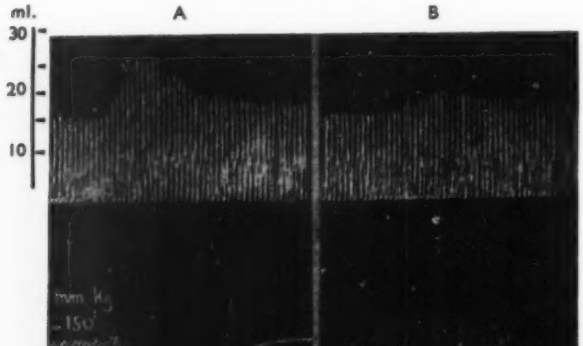

FIG. 4.—Cat, 1.8 kg.; chloralose. Records as in Fig. 2. Intravenous injection of 15 mg. diphenhydramine. Between A and B both cervical vago-sympathetic trunks were cut. The tidal air is somewhat reduced even after cutting the vagi.

Diphenhydramine.—Diphenhydramine (10–20 mg.) also caused a reduction in tidal air (20 cats, Fig. 4), but it was usually larger than that due to 2-α-naphthyl ethyl isothiourea, and could be obtained in unhealthy animals which were not sensitive to the latter compound. The effect was still obtained after section of the vagi and the cervical sympathetic in 11 cats, and there was still a fall of blood pressure. The reduction in tidal air was only obtained on intravenous injection and not after injection into the left atrium, and the left atrial pressure rose after injecting diphenhydramine. Large effects were still obtained after atropine. The latent period was often considerably greater than for the isothiourea. It is interesting that the effect of this compound on tidal air appears to be direct rather than reflex, for in other respects the pulmonary reflexes due to amidines and antihistamines were found to be similar in the cat (Jones, 1952). The effect may, however, be more complicated than at first appears, since after eserine diphenhydramine caused increases in tidal air in some cats, which were abolished by cutting the vagi.

Peptone.—It was decided to see if these reflexes could be excited by a substance known to produce anaphylactic symptoms. Bacteriological peptone in doses of 100–200 mg. produced a very large decrease in tidal air and a fall in blood pressure (five cats), but this was not abolished by section of the vagus and sympathetic (two cats), and no reflex inhibition of natural respiration (one cat) was obtained. The effect is probably a direct bronchoconstriction similar to that caused by histamine.

Embolism.—Emboli were produced by the intravenous injection of either air (5 ml.) or chlorbismol (0.5 ml.). Both produced a considerable decrease in tidal air which was not abolished by section of the vagus and sympathetic. One would suspect that pulmonary congestion due to obstruction by emboli was the cause of the displacement of air, but bronchoconstriction cannot be excluded. Air emboli but not chlorbismol produced a rapid fall in blood pressure.

Veratrine.—The action of veratrine (20–100 μg.) on tidal air was investigated in 17 cats and found to be variable. A considerable decrease in tidal air was found in four cats, a very small decrease in seven cats, and no change in four. In two cats (one had received eserine) the reverse effect, an increase in tidal air, was observed after 100 μg. veratrine (Fig. 5). The vagi and sympathetic were cut in eight cats, with results which depended on the initial response to veratrine. One cat which showed a large decrease in tidal air before vagotomy gave a large response afterwards; a second which had shown moderate decreases gave a very small decrease afterwards; two cats which had given very small decreases before section gave none afterwards (possibly because of tachyphylaxis), while two showed no responses either before or after nerve section. The two cats in which the reverse effect, an increase in tidal air, was obtained showed no response after cutting the vagus and

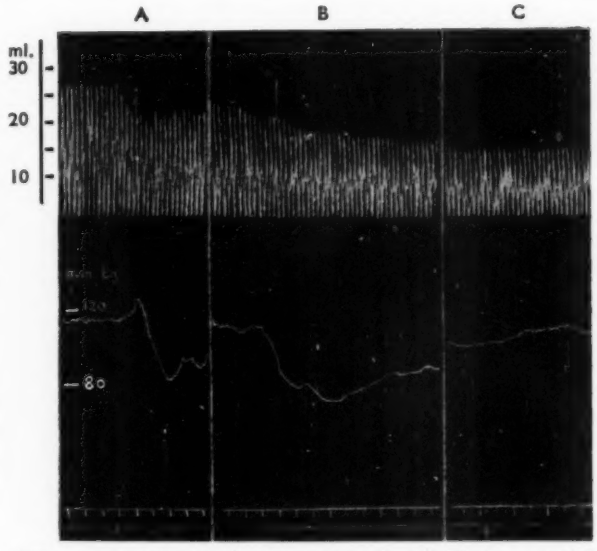

FIG. 5.—Cat, 3.7 kg.; chloralose. Records as in Fig. 2. A and C 100 μg. veratrine intravenously. At B the cervical vago-sympathetic trunks were cut.

sympathetic. Since the results are so variable it is not possible to say with certainty whether veratrine causes these changes in tidal air by reflex or direct action, but they suggest that the decrease in tidal air may be a direct action (confirming Mosey and Kaplan, 1952), while the increase is reflex. The changes in breathing and blood pressure are distinct, since they varied in size independently, and the latter was abolished by section of the vagus and sympathetic.

Discussion

All the pulmonary reflexes studied were shown to be accompanied by a decrease in tidal air, but only after 2-α-naphthyl ethyl isothiourea was the decrease of tidal air itself reflex in nature, and even there the pathway involved appeared complicated. After diphenhydramine and veratrine both decreases and increases in tidal air were recorded and only the latter were possibly reflex.

It has been emphasized that it is difficult to be certain whether the decreases in tidal air are due to bronchoconstriction or pulmonary congestion either with the method we used or with other methods. Theoretically the direct measurement of alveolar pressure is the only sound approach to the problem, but the practical solution of this method presents great difficulties. Vuilleumier (1944) tried to measure alveolar pressure indirectly by measuring pressure in the mouth during a sudden interruption of respiratory movement; the respiratory tract was assumed to reach an equilibrium pressure equal to that in the alveoli. However, the time intervals involved are extremely small, and no true equilibrium is attained, since the lungs continue to move.

For 2-α-naphthyl ethyl isothiourea, the balance of evidence was in favour of bronchoconstriction being responsible for the decrease of tidal air. The size of the response did not seem to be related to the degree of vascular disturbance in the lung, and an effect was still obtained when congestion was minimized by bleeding from the left atrium. In the presence of a blood pressure stabilizer the isothiourea caused a still larger decrease of tidal air on the other hand, and infusion of fluid into the lesser circulation produced definite decreases in tidal air. The effects of infusion were, however, never very large, and this procedure was not physiological, since large volumes of blood or saline (20–40 ml.) were rapidly injected intravenously or into the right side of the heart. It is hard to believe that such large blood volume changes could occur in the lung in the short interval before the tidal air decreases.

The reduction in tidal air after diphenhydramine must be due either to direct action on the bronchial muscles or to an increase in pulmonary blood volume. One point in favour of the latter was that repeated responses to diphenhydramine were obtained in an animal which failed to show a bronchoconstrictor response to histamine acid phosphate (200 μg.).

Attention has been drawn by many workers to the effect of pulmonary congestion on ventilation (Drinker, Peabody, and Blumgart, 1922; Christie and Hayward, 1943), and this has been suggested as the cause of the dyspnoea and decreased vital capacity of heart disease. Compression of the pulmonary veins caused a twofold increase of the lung blood volume in heart-lung preparations in cats (Drinker, Churchill, and Ferry, 1926), while the right pulmonary artery was able to accommodate a normal blood flow for the whole lung when the left was occluded.

It is clear, therefore, that the lungs can accommodate a large volume of blood. From the practical point of view the effect on the subject of bronchoconstriction or pulmonary congestion could be very similar. One observation was made during the present work which may be worth mentioning here. Adrenaline sometimes caused a decrease and sometimes an increase in tidal air. Both effects were present after cutting the vagi, and the decrease was sometimes abolished by preventing the blood pressure rise with a stabilizer. The decrease may therefore be due to accumulation of blood in the lungs, since when a stabilizer is in operation a considerable volume of blood flows out into it. This decrease in tidal air was often quite large, and it either completely masked the direct bronchodilator action of adrenaline or was followed by a brief dilatation.

Summary

1. The action of 2-α-naphthyl ethyl isothiourea, diphenhydramine, veratrine, peptone, and embolism on the tidal air was studied.

2. All these agents decreased the tidal air under certain circumstances. Only the decrease caused by 2-α-naphthyl ethyl isothiourea was abolished by cutting the vagi.

Diphenhydramine and veratrine occasionally caused increases in tidal air, an effect which was probably reflex.

3. Measures which increased the volume of blood entering the lungs decreased the tidal air, both before and after cutting the vagi. It was not always possible to decide whether a decrease in tidal air

was due to an alteration in bronchial tone or to an increase in pulmonary blood volume.

We wish to record our gratitude to Dr. G. S. Dawes for his help and advice in this work, and to Dr. W. F. Short for the 2-α-naphthyl ethyl isothiourea hydrochloride.

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THE TECHNIQUE OF SUPERFUSION

BY

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The amount of an active substance required for an assay on isolated plain muscle suspended in a bath depends upon the volume of the bath, which cannot conveniently be made much smaller than about 2 ml. A piece of intestine may be suspended in air and kept in good condition by a stream of a suitable solution running over its surface (Finkleman, 1930). This technique may be called superfusion, since the fluid runs over the tissue, by analogy with perfusion, in which the fluid runs through the tissue.

The effect of drugs on such a preparation may be tested by injecting them into the stream of fluid (Gaddum, Jang, and Kwiatkowski, 1939), and such preparations have been used for the assay of histamine (Kwiatkowski, 1941).

Greater sensitivity can be obtained by stopping the flow for a standard interval of time and applying the active solutions undiluted to the surface of the muscle. The object of the present paper is to describe an apparatus which has been developed during the last few years for this purpose and to present some results obtained with it.

METHODS

The apparatus is shown in Fig. 1. The muscle is suspended in air in the middle of a wide glass tube which is immersed in warm water in a small copper bath. This copper bath is attached to a vertical rod and the lever is attached to the same rod. The warm water is mixed by means of a stream of air which is sufficiently violent to cause slight vibration of the whole apparatus and so diminish friction of the writing point on the drum. The vibration may be controlled by finding the appropriate height for the apparatus on the vertical bar. The appropriate salt solution is contained in a reservoir 20–40 cm. above the bath. It runs from this reservoir through a glass tap and a glass-wool filter, and is then warmed by passing through the water in the copper bath. The rate of flow is controlled by a resistance which consists of a capillary tube drawn out to a fine point and held inside a wider glass tube by passing the same rubber tube over both. The flow is adjusted to 1–5 ml./min. by altering the height of the reservoir. The superfusion fluid drips from a glass tube down the cotton which attaches the tissue to the lever. The end of this tube has been filed off at an acute angle in order to facilitate the administration of drugs. The height of the glass tube above the tissue must not be too small, or the solution may run down one side of the tissue and allow the other side to dry. If the height is sufficient (> 10 cm.), each drop is broken up on reaching the tissue, and wetting is even.

Solutions to be tested are contained in small test-tubes (12×65 mm.) which are warmed before use by placing them in a suitable holder attached to the copper bath.

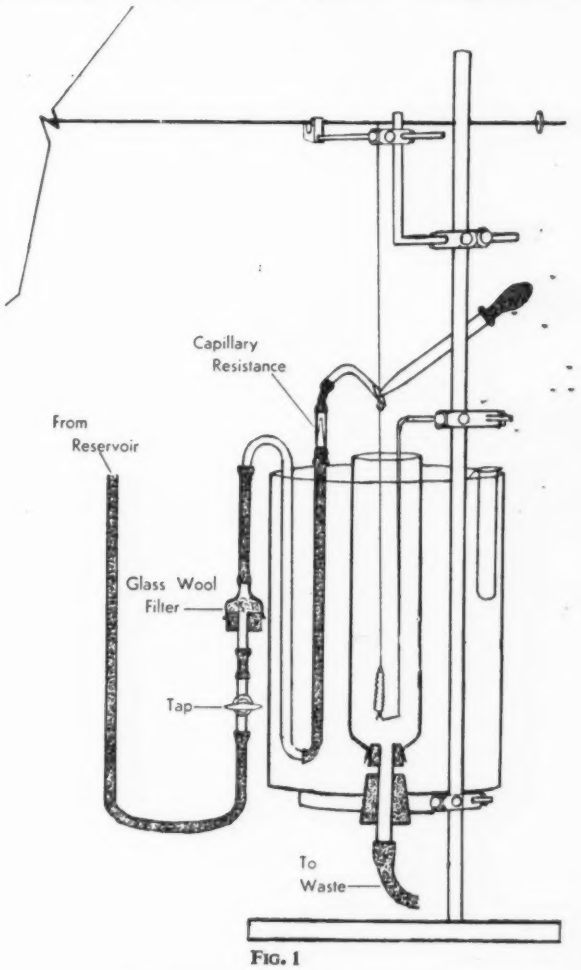

FIG. 1

A small volume of this fluid is sucked into a teat pipette. The tap which controls the flow is then turned off for a standard time. The fine point of the teat pipette is placed in the filed-off end of the glass tube which forms the drop, and the teat is squeezed until a standard number of drops (generally 5 drops or about 0.2 ml.) has fallen; this takes 3–4 seconds. These drops are formed in a standard place in the angle between the thread and the glass tube, so that their size does not depend on the pipette, but their exact volume is not critical. The fluid is left in contact with the surface of the tissue for a standard time (10–60 sec.), and the tap is then opened and the normal flow restarted.

The effect of a drug depends on its concentration in the solutions tested, and it may be necessary to prepare a large number of different dilutions. This may be done quite rapidly by measuring out 0.2–1 ml. of a more concentrated solution into the small test-tubes with a 1-ml. measuring pipette and adding the appropriate volume of the superfusion fluid to make 1 ml. In this case the more concentrated solution must be very similar in *p*H and salt content to the superfusion fluid. Since fluids are applied direct to the tissue, small differences in their composition may affect the result. For example, superfusion fluid diluted with only 5% of water has been found to cause a contraction of guinea-pig's ileum.

In the experiments on guinea-pig's ileum the superfusion fluid was Tyrode's solution and the temperature of the bath was 30°–37° C. In the experiments on rat's uterus the fluid was de Jalon's solution (Gaddum, Peart, and Vogt, 1949), and the temperature was 25°–30° C. The magnification on the lever was 5—or sometimes more. The lever was adjusted to give a tension of 0.5–1 g. wt., which is partly counterbalanced by the weight of the muscle in air.

A modified form of the apparatus described above was used for experiments in which two tissues were suspended one above the other and the same fluid was superfused over them both in series (cf. Finkleman, 1930). In this way it was found that a substance was sometimes liberated from rat's small intestine which caused a contraction of rat's uterus. This observation led to experiments in which the rat's small intestine was perfused with Tyrode's solution at 37° C. through a polythene cannula tied in the superior mesenteric artery. All other vessels were tied except the portal vein, which was left open; both ends of the small intestine were tied. The tissue was suspended in a gauze hammock fixed in a funnel which was warmed in a bath; the outflow was collected from the funnel. The reservoir was at a height of about 50 cm. above the intestine and the rate of flow was kept constant (0.1–0.5 ml. per min.) by means of a capillary resistance.

Rats' uteri were generally prepared by the injection of stilboestrol (0.1 mg. per kg.) the day before the experiment, but this is not essential.

Crystalline preparations of trypsin and chymotrypsin containing less than 50% $MgSO_4.7H_2O$ were obtained from Messrs. Armour. Oxytocin was used in the form of Pitocin (Parke, Davis). The preparation of substance P (P_s) was made by Drs. A. H. Amin and T. B. B. Crawford by the method of Euler (1942). It was compared with a preparation obtained from Professor Euler and was estimated to contain 13.8 of his units per mg.

Results

Stoppage of the flow may itself cause contraction of superfused muscle which is probably due, sometimes at least, to the liberation of active substances which remain on the surface of the muscle. This effect is well shown by guinea-pig's ileum, and with this tissue it may be necessary to restrict the time of stoppage to 10–15 sec. This effect is partly due to choline and choline esters, and may be diminished by adding atropine to the fluid (Magnus, 1930), but some effect survives in

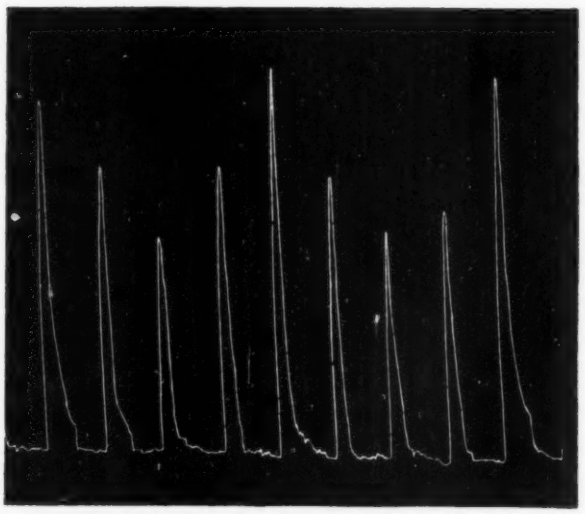

FIG. 2.—Guinea-pig's ileum superfused. Approximate assays of four unknown solutions (A–D) in terms of histamine base (H—μg./l.).

atropine. Rat's uterus does not contract so readily when the flow is stopped.

Fluids are normally tested by applying 5 drops (ca. 0.2 ml.) in a short time (3–4 sec.). The fluid on the surface of the tissue is thus almost completely replaced, and this new fluid is now given a standard time to act. If more than 5 drops are used the effect may be slightly larger, but if less than 5 drops are used the effect may be much less, owing to incomplete replacement of the fluid on the surface of the tissue. Five drops has appeared to be a reasonable compromise between the conflicting claims of the accuracy which might be achieved with more drops and the economy which would be achieved with fewer.

When histamine is applied to guinea-pig's intestine the contraction of the muscle generally begins in less than 15 sec., but does not reach its maximum until about 20–30 sec. after the application of the drug when the muscle has already been superfused with drug-free fluid for some time. With slow-acting substances, such as trypsin and the substance R whose properties are discussed later in this paper, there may be no response until 30 sec. or more after the washing out of the drug has started, and the maximum may not be reached till a minute or two later (Fig. 3). These unconventional time-relations appear to be compatible with accurate assays. This preparation is, in fact, particularly suitable for the study of slow-acting drugs, since it is unnecessary to expose the tissue to the possibly deleterious effect of the drugs for long periods while slow effects are actually recorded on the drum. The muscle records its response some time after the drug has been washed away.

The Assay of Histamine

The concentration of histamine which causes a contraction of superfused guinea-pig's ileum is similar to the final concentration in the bath when more conventional methods are used. The sensitivity of the method is thus similar to that which might be achieved with a bath-volume of 0.2 ml. Kwiatkowski (1941) has already emphasized the value of a method not unlike that used here, but his method is probably less sensitive because the histamine is more diluted and has less time to act.

In the assay shown in Fig. 2 the drugs were applied for 10 sec. at intervals of 2 min. Small concentrations of histamine (1–10 μg./l.) caused fairly regular effects. The experimental design used here gives a quick result. The unknown solutions alternate regularly with the standard solution which is itself used in two different doses alternately. The concentration of the unknown is chosen so as to cause effects intermediate between the effects of the two different doses of the standard, which precede and succeed it. The potency of the unknown is then calculated on the assumption that the effect is linearly related to log dose. The first three assays shown in Fig. 2 illustrate this design. In the fourth assay the sequence of doses was slightly different. Two estimates of the histamine concentration were made from each of these four assays, and the four duplicate results were 2.8, 2.6; 2.6, 2.2; 1.5, 1.6; and 1.7, 1.8 μg./l. These figures illustrate the fact that the superfused intestine behaves with reasonable consistency, but the two estimates in each pair are not independent, and the error of an assay is likely to be greater than that calculated directly from the differences between the duplicates.

Substance R

Tyrode's solution, or de Jalon's solution, which had been perfused through the rat's intestine by the method described above, caused a slow and delayed contraction, when applied to a superfused rat uterus in the presence of atropine (10^{-6}). Samples collected in the first hour were not very active, but during the next 3–6 hours their activity was high and fairly constant. During this time they were generally still effective when diluted 20–50 times with the superfusion fluid. The substance responsible for this effect will be called substance R for convenience in this discussion.

It was soon found that substance R was precipitated by mixing the perfusion fluid with twenty volumes of cold acetone, and dry standard preparations were made in this way. These preparations (R_1–R_5) contained much inorganic salt, but were reasonably active and were used in most of the experiments. Later it was found that substance R could be precipitated quantitatively by adding only four volumes of cold acetone to the effluent and allowing the mixture to stand in the cold. A preparation made in this way was about five times more active than the earlier preparations

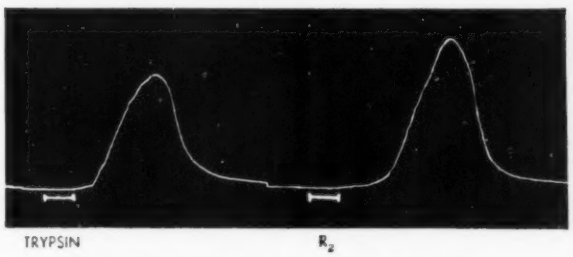

FIG. 3.—Rat's uterus superfused. Atropine 10^{-6}. Slow responses to crystalline trypsin (20 mg./l.), and R_2 (50 mg./l.) applied for 30 sec. at the signal.

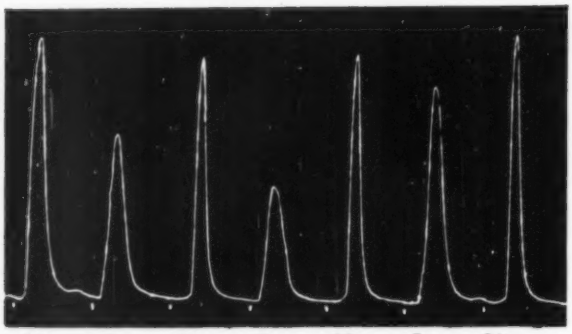

FIG. 4.—Rat's uterus superfused. Atropine 10^{-6}. Application 30 sec. (except 60 sec. at T*). The effect of crystalline trypsin (T) was irregular, but less than the effect of half the weight of R_6. R_2 = approx. 1/5 R_6.

(Fig. 4), but none of these preparations gave a clear solution in water.

Fig. 3 shows the effect of the preparation R_2 recorded on a fast drum to show the slow way in which the response develops. This figure also shows the response to a crystalline preparation of trypsin, which caused a similar slow response. This action of trypsin was described by Rocha e Silva (1939) in a comprehensive paper on the pharmacology of this substance. The response to substance R or trypsin may be obtained repeatedly, and in good preparations constant effects may be recorded at intervals of 5 min. Assays can be made by altering the concentrations, and these are most conveniently recorded on a slow drum.

The results shown in Fig. 4 prove that the preparation R_6 was more active weight for weight than a crystalline preparation of trypsin. A crystalline preparation of chymotrypsin also caused a slow effect like that due to trypsin, but was about 10 times less active.

Substance R does not dialyse through cellophane. In one experiment more than 50% of the original activity was recovered when a solution had been dialysed against running tap water for 21 hr. In another experiment a solution was dialysed for 21 hr. against an equal volume of de Jalon's solution by shaking it in a dialyser like that used by Verney (1926). After this time no activity was detected in the de Jalon's solution and no loss in the original solution.

Neutral solutions of substance R appeared to be stable for at least several hours at room temperature, but were partly destroyed by boiling for 3–5 min. At *p*H 2 the activity disappeared quite rapidly at room temperature.

Substance R was not destroyed by trypsin or chymotrypsin. A solution containing R_3 (5 mg./ml.) and crystalline trypsin (0.5 mg./ml.) was incubated for 1 hr. at 37° C. and then boiled for 3 min. to destroy the enzyme. A similar solution containing boiled trypsin was incubated at the same time and also boiled for 3 min. after the incubation. The amount of activity found in both of these solutions was between 30 and 40% of the original activity. The loss was presumably due to the boiling; the trypsin had no apparent action. Identical results were obtained with chymotrypsin. On the other hand, a preparation of substance P was completely inactivated by both these enzyme preparations in a few minutes (Amin, 1953). These results suffice to show that substance R is not readily destroyed by trypsin or chymotrypsin.

Substance R is not histamine, because histamine does not cause contraction of rat's uterus. It is distinguished from acetylcholine and 5-hydroxytryptamine (HT) by its failure to dialyse, and the slowness of its action, and by the fact that this action survives in the presence of atropine (10^{-6}) or lysergic acid diethylamide (10^{-7}). The latter substance has been found to anatagonize HT on rat's uterus (Gaddum, 1953). Fig. 5 shows that it abolished the effect of HT completely without affecting the response to the preparation R_6.

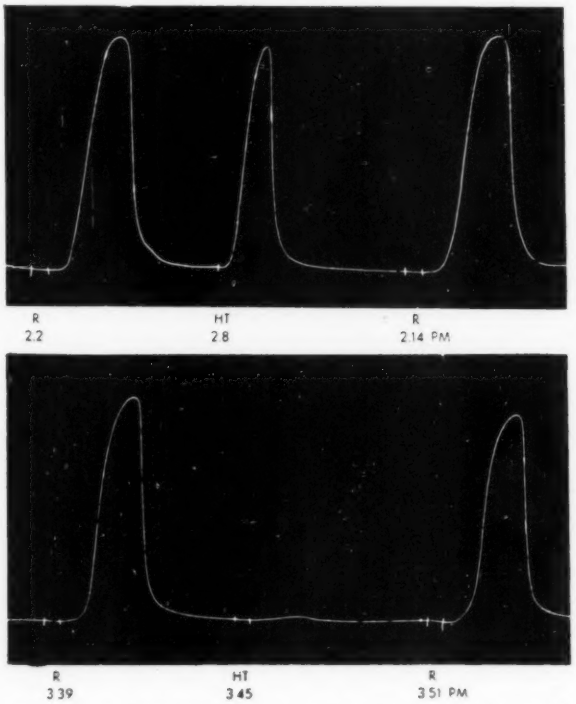

FIG. 5.—Rat's uterus superfused. Atropine 10^{-6}. R—R_6 (20 mg./l.); HT—5-hydroxytryptamine (100 μg./l.). Application for 30 sec. at times marked on record. Response to R relatively slow. From 3.26 p.m. Lysergic acid diethylamide (10^{-7}); this abolished the response to HT, but not that to R_6.

Bradykinin (Rocha e Silva, 1951) differs from substance R because it is dialysable and destroyed by chymotrypsin.

The possibility was considered that substance R might be identical with oxytocin, which is known to stimulate rat's uterus in low concentrations (Holton, 1948). This possibility is excluded by the results shown in Figs. 6 and 7. Fig. 6 shows that oxytocin had a good effect in a concentration of 100 μU./ml. (total dose about 25 μU.) and that this was similar to the effect of R_2 in a concentration of 0.6 mg. per ml. Both these active solutions were then treated with thioglycollate by the method recommended by Ames, Moore, and van Dyke (1950) for the specific inactivation of posterior pituitary extracts. Oxytocin was almost completely inactivated in 38 min., and R_2 survived for 56 min. with its activity at least as great as it had been originally.

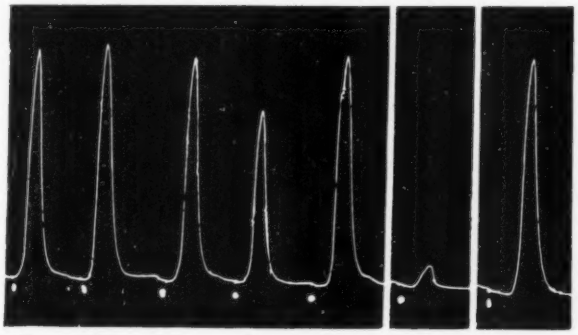

FIG. 6.—Rat's uterus superfused. Atropine 10^{-6}. Treatment with thioglycollate destroys oxytocin, but not substance R. R—R_2 (60 mg./l.); RT same dose treated 56 min. OX—oxytocin (mU./ml.); OXT 0.2 mU./ml. treated 38 min.

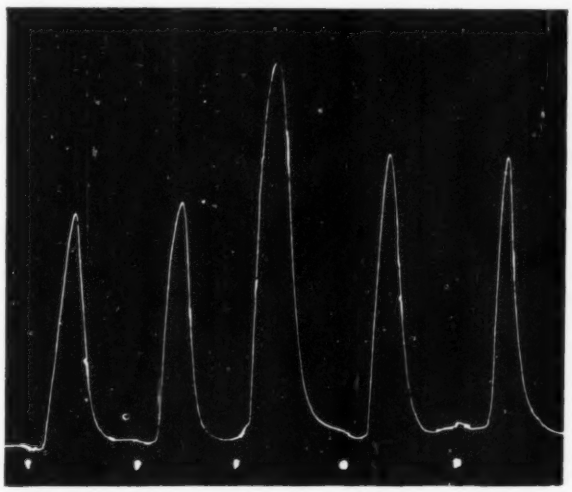

FIG. 7.—Rat's uterus superfused. Atropine 10^{-6}. R—R_2 (250 mg./l.); ROX—the same plus oxytocin (1 mU./ml.). ROXT—ROX treated with thioglycollate from 2.37 p.m. Thioglycollate was effective in destroying oxytocin in the presence of R_2.

It might be suggested perhaps that R_2 contained some substance which inhibited the action of thioglycollate. In the experiment shown in Fig. 7 oxytocin and R_2 were therefore mixed in roughly equivalent amounts and the mixture was treated with thioglycollate. The results show that the mixture was partially inactivated and that the remaining activity was equivalent to that of the preparation R_2 which had been used. This shows that thioglycollate was effective in the presence of R_2, and is consistent with the view that it has no action on substance R.

Substance R is distinguished from substance P by the fact that it does not dialyse and is not destroyed by trypsin or chymotrypsin. It is also distinguished by the fact that it has comparatively little effect on the guinea-pig's ileum. The preparations R_2 and P_6 were compared with one another by their actions on guinea-pig's ileum in a bath in the presence of mepyramine. The comparison was difficult, since the effects were dissimilar, but, as judged by the immediate effect, the activity of R_2 appeared to be about 0.3% of that of P_6. This effect may have been due to a small amount of substance P in the preparation R_2. These two preparations were then compared for their effects on superfused rat's uterus. When the maximum effects were similar, the latent period for R_2 was longer than that for P_6. An assay based on the maximum contraction led to the conclusion that the activity of R_2 was now about 50% of that of P_6—a result very definitely larger than that obtained on the guinea-pig's ileum (0.3%). This shows that the active substances in R_2 and P_6 are different.

In another experiment the whole of a rat's small intestine and contents were cut up in acetone, and the insoluble residue was extracted with acid. This extract was compared with the standard preparations P_6 and R_2 by its action on guinea-pig's ileum in a bath in the presence of mepyramine and atropine. It was also compared with these two standard preparations by its action on superfused rat uterus. The results are shown in Table I.

TABLE I

AMOUNT OF ACTIVITY IN THE WHOLE SMALL INTESTINE OF A RAT

Using Assay	Guinea-pig Ileum	Rat Uterus	Ratio Ileum/Uterus
P_6-equivalent, mg. ..	3·3	85	1/26
R_2- ,, ,, ..	1,150	170	6·8
Ratio mg.R_2/mg.P_6 ..	350	2	175

The discrepancy between the two estimates of the P_6-equivalent is evidence that substance P was not the only substance present in the extract. The discrepancy between the two estimates of the R_2-equivalent is evidence that substance R was not the only substance present. The results are compatible with the view that the experiment with guinea-pig's ileum gave a reasonably good estimate of the amount of substance P in the extract and that the experiment with the rat's uterus gave a reasonably good estimate of the amount of substance R. The consequences of this assumption can be calculated by considering the figures in the bottom line of Table I. These show, for example, that P_6 was 350 times as active as R_2 on the ileum. The P_6 equivalent of the amount of substance R assumed to be present is thus 170/350 mg., or about 0.5 mg., which is almost negligible compared with 3.3 mg. This is a maximum estimate of the effect of substance R in this test, based on the assumption that the preparation R_2 contained no substance P. If R_2 did contain substance P, the figure 350 is too low and the figure 0.5 mg. is too high. It is thus unlikely that substance R seriously interferes with this test for the estimation of substance P except when the proportion of substance R present is even higher than in this experiment.

In the same way P_6 was twice as active as R_2 on rat's uterus. The R_2 equivalent in this test of the amount of substance P which is assumed to be present is thus 3.3×2, or 6.6 mg., which is negligible compared with 170. Substance P is therefore unlikely to interfere with the use of the superfused rat's uterus for the estimation of substance R.

Estimates were made by this method of the amount of substance R in different parts of the rat's intestine. The small intestine was roughly divided into three fractions consisting of contents, mucous membrane, and muscle. The contents contained more than the mucous membrane, and the muscle contained little. Activity was also found in the contents of the colon, but not in the contents of the stomach. These data need amplification, but they suggest that substance R may be secreted by some gland into the small intestine.

Discussion

The technique described here is sensitive, and especially suitable for the study of slow-acting substances. The study of substance R by more conventional methods would be more troublesome. This substance cannot be histamine, acetylcholine, 5-hydroxytryptamine, oxytocin, or substance P. The only substances found to have an action as slow as that of substance R were trypsin and chymotrypsin. The fact that a simple acetone precipitate was actually more active than crystalline preparations of these enzymes suggests that substance R is not trypsin or chymotrypsin, but more evidence is required. The known properties of substance R are similar to those of kallikrein (Werle and Berek, 1950). The possibility that these two substances are the same has not yet been fully explored. However this may be, the fact remains that substance R may appear as a complication of experiments on the pharmacological activity of extracts of intestine, or of experiments designed to detect the release of active substances by the intestine under various experimental conditions.

Summary

1. The word superfusion is used to describe experiments in which suitable liquids run over tissues suspended in air. A new method of applying drugs to such tissues is described.

2. The value of superfused guinea-pig's ileum for the assay of histamine is confirmed.

3. Superfused rat's uterus may be used in assays involving oxytocin, the effective dose of which is about 0.02 milliunits.

4. Superfusion is particularly suitable for the study of slow-acting substances. This is illustrated by the analysis of the properties of " substance R," which comes from rat's intestine and causes a slow contraction of rat's uterus. This substance does not dialyse through cellophane, and is insoluble in 80% cold acetone and rather unstable in watery solution. Its properties resemble those of some proteolytic enzymes, but its identity has still to be established.

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Brit. J. Pharmacol. (1953), **8**, 327.

THE ACTION OF ACETYLCHOLINE ANTAGONISTS ON THE HEART OF *VENUS MERCENARIA**

BY

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An attempt has been made to gain a better understanding of the structure-activity relationships of acetylcholine and one type of "receptor substance" by restricting attention to one suitable, isolated organ, the ventricle of the heart of the mollusc, *Venus mercenaria* L. (Welsh and Taub, 1948, 1950, 1951). It has been noted that quaternary ammonium compounds must have two or more methyl groups on a given nitrogen if they are to act like acetylcholine on this preparation. Related compounds with three groups other than methyl (e.g. ethyl or *n*-propyl) on the nitrogen are antagonistic to acetylcholine. These and other earlier observations on the requirements for blocking action led to a more extensive comparison of the relative activities of a number of known and potential acetylcholine antagonists on the Venus heart. From the results that follow it may be seen that the general pattern of action differs from that found at any of the better-known sites of action of acetylcholine in the vertebrates, such as synapses in autonomic ganglia, or neuromuscular junctions. What new light, if any, this throws on acetylcholine receptor substances will be discussed.

METHODS

The procedure used in isolating the ventricle preparation and applying the drugs was the same as previously described (Welsh and Taub, 1948). The steps followed in determining the activities of several concentrations of a given antagonist may best be set forth by reference to a given experiment. In Fig. 1 is shown a record of the blocking action of four different molar concentrations (10^{-6} M to 10^{-3} M) of 4-ketoamyltriethylammonium iodide (K-4). First, by successive trials (only the last of which is shown) a concentration of acetylcholine producing between 20 and 80% decrease in amplitude of heart beat was found. In the illustrative case 10^{-5} M acetylcholine bromide was found to produce 76% decrease in amplitude. After stopping the drum and washing until the original amplitude was regained (3–5 minutes), an amount of 4-ketoamyltriethylammonium iodide was added to produce a final concentration of 10^{-6} M in the bath. Two minutes later 10^{-5} M acetylcholine was again added. Now it was found to produce only 30% decrease in amplitude. From this it was calculated that the activity of the acetylcholine was antagonized or blocked to the extent of 61%. This assumes that repeated applications of a given concentration of acetylcholine, if separated by thorough washing, would have a constant effect, which we know is ordinarily true.

This procedure was then repeated with higher concentrations of 4-ketoamyltriethylammonium iodide up to and including 10^{-3} M, with results as indicated by Fig. 1. From 6 to 20 different heart preparations were used in determining the activity of each compound, and the results were averaged. Many compounds showed no blocking action at a concentration of 10^{-6} M, in the time they were allowed to act on the heart, therefore the tables of results include only the three higher concentrations of antagonists normally tested. With one compound, viz., "Mytolon," the antagonistic action was so much greater that a range of concentrations from 10^{-5} to 10^{-6} M was employed. Certain compounds having two or three methyl groups on the nitrogen acted like acetylcholine in low concentrations, but were antagonistic at higher concentrations. Attention will be called to these when the results are presented and discussed.

We are greatly indebted to the sources indicated for gifts of the following materials:

d-Tubocurarine chloride (crystalline), E. R. Squibb & Sons
Pentamethonium bromide, Decamethonium bromide } The Wellcome Research Laboratories
RO 2–2561, RO 2–3198, RO 1–3724/N1 } Hoffman-LaRoche, Inc.
"Banthine," G. D. Searle & Company
"Mytolon" (WIN 2747), Sterling-Winthrop Research Institute.

The other compounds used were commercial products, with the exception of 4-ketoamyltriethylammonium iodide and chloride, which were synthesized for the first time using the same general procedures

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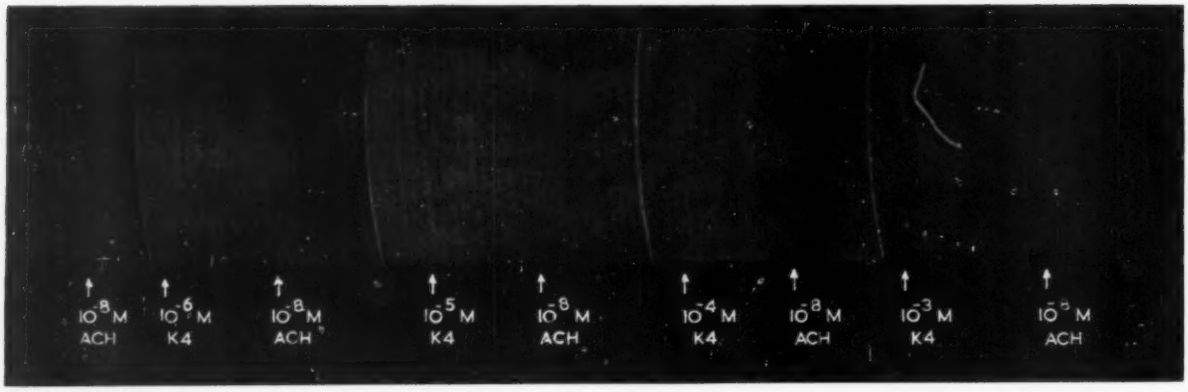

FIG. 1.—Record of the antagonism between 4-ketoamyltriethylammonium iodide (K-4) and acetylcholine bromide (Ach), using the response of the isolated ventricle of the heart of the mollusc, *Venus mercenaria*. The per cent decreases in amplitude produced by 10^{-8} M acetylcholine alone and in the presence of four different molar concentrations of K-4 are shown. From these the percentage block is calculated.

described earlier (Welsh and Taub, 1951). Ing, Kordik, and Tudor Williams (1952) also give methods for preparing certain ketone analogues of acetylcholine.

RESULTS

Table I summarizes the results that were obtained. The grouping of the compounds, although somewhat arbitrary, will facilitate discussion. A number of known acetylcholine antagonists were not tried chiefly because time limitations prevented further study.

Group A.—Included here are three of the oldest known acetylcholine blocking agents. Although acetylcholine has a negative inotropic and chronotropic action on the mollusc heart, it has frequently been observed by other workers (cf. Fredericq, 1947) that atropine fails to block the acetylcholine action as it does in the vertebrate heart. We had stated (Welsh and Taub, 1950) that atropine was completely lacking in acetylcholine blocking action on the Venus heart. However, we observed in this study that atropine was slightly antagonistic at 10^{-5} and 10^{-4} M but quite effective at 10^{-3} M. We had also failed earlier to find any consistent action of *d*-tubocurarine on the Venus heart. Now, after more extensive observation, it appears to potentiate the action of acetylcholine slightly at 10^{-5} and 10^{-4} M and to have a weak blocking action at 10^{-3} M concentration. Luduena and Brown (1952) observed a slight potentiation of acetylcholine with 3.08×10^{-4} M *d*-tubocurarine.

We had noted (Welsh and Taub, 1950) that nicotine in concentrations of 10^{-7} and 10^{-6} M had an acetylcholine-like action on the Venus heart, while at higher concentrations it blocked acetylcholine.

TABLE I

RELATIVE ACTIVITIES OF ACETYLCHOLINE BLOCKING AGENTS ON THE VENUS HEART

(Values indicate the percentage block, by the indicated concentrations of the blocking agents, of amounts of acetylcholine producing 20–80% decrease in amplitude. Each value is the average of 6 to 20 determinations on different hearts)

	10^{-5} M	10^{-4} M	10^{-3} M
Group A:			
Atropine sulphate	10	6	74
d-Tubocurarine Cl	*	*	48
Nicotine	10	60	80
Group B:			
Pentobarbital sodium	18	36	54
Phenobarbital ,,	18	50	48
Cocaine HCl	22	18	88
Tropacocaine HCl	—	18	48
Procaine HCl	0	0	64
Group C:			
Pentamethonium Br	0	0	*
Decamethonium Br	0	10	66
Group D:			
n-Octyltrimethylammonium Cl	*	*	40
Phenyltrimethylammonium I	24	60†	90†
3-Hydroxyphenyltrimethylammonium Br=RO 2–2561	[67]	[92]	[100]
3-Hydroxyphenyldimethylethylammonium Cl=RO 2–3198 or "Tensilon"	12[17]	[77]	[100]
Group E:			
"Banthine" or methantheline Br	4	54	100
4-Ketoamyltriethylammonium I	44	70	88
Phenyltriethylammonium I	40	90	100
3-Hydroxyphenyltriethylammonium I=RO 1–3724/N1	54	84	100
	10^{-8} M	10^{-7} M	10^{-6} M
"Mytolon" or WIN 2747	40	78	100

* Potentiation of the acetylcholine response or slight Ach-like action.

† Some acetylcholine-like action of the compound alone followed by effective blocking action.

[ ] Values in brackets are the percentage inhibitions of heart beat produced by the compound alone. See text.

It is a more effective antagonist at 10^{-4} M than atropine or *d*-tubocurarine. Since, in addition, pilocarpine has little or no significant action on the Venus heart we had earlier suggested that the action of acetylcholine on this organ could be described as "nicotine-like" and most closely resembling that found at vertebrate autonomic ganglia. We were aware from our own results that this view required modification before it was pointed out by Luduena and Brown (1952). This will be given further attention in the discussion.

Group B. (1) Barbiturates.—The mechanism of action of the barbiturates is in dispute. One possibility that has received little attention is that they block acetylcholine, especially at certain points within the central nervous system. After recognition of the importance of the C=O group as one of the attaching groups between acetylcholine and receptor substance of the Venus heart (Welsh and Taub, 1951) the question of the significance of C=O groups in so many of the hypnotics, local anaesthetics, and anti-epileptics arose. If bis-quaternary ammonium compounds make such effective acetylcholine antagonists through the formation of double ionic linkages with acetylcholine receptor substance, might not certain compounds with two or three carbonyl groups (e.g. ureides, hydantoins, barbiturates) form multiple hydrogen bonds with receptor substance? In attempting to answer this question, we have done little more, thus far, than independently confirm the observations of Fernando (1952) that certain barbiturates do block the action of acetylcholine on the Venus heart. Fernando found sodium diethyl barbiturate and sodium phenobarbital to be moderately effective in blocking acetylcholine. We found a similar action of phenobarbital and noted, in addition, that sodium pentobarbital has about equal activity with the phenyl derivative.

Group B. (2) Cocaine, Tropacocaine, Procaine.—Thimann (1943) appears to have been the first to suggest that the actions of certain local anaesthetics such as cocaine, tropacocaine, and procaine might be accounted for on the basis of structural resemblance and competition with acetylcholine. There is evidence in the literature indicating that this is true (e.g. De Elío, 1948). These compounds react with cholinesterase (e.g. cocaine, Blaschko, Chou, and Wajda, 1947). Perlmutter (unpublished), using a variety of isolated smooth muscle preparations from invertebrates, has found (*a*) an acetylcholine-like action, (*b*) potentiation of acetylcholine through block of cholinesterase, and (*c*) acetylcholine antagonism, depending on the particular preparation and the concentration of cocaine that was applied.

On the Venus heart 10^{-5} M cocaine has a low blocking activity. Over a very narrow range (around 10^{-4} M) cocaine acts like acetylcholine, yet at the same time it is capable of antagonizing acetylcholine (Fig. 2). 10^{-3} M cocaine causes a striking increase in amplitude of heart beat partly, if not wholly, through the blocking of endogenous acetylcholine, and at this concentration is very effective as an antagonist to applied acetylcholine.

Tropacocaine acts on the heart in a manner similar to cocaine, but at 10^{-3} M concentration it is less active as an acetylcholine antagonist. Procaine, on the other hand, has no demonstrable effect on the acetylcholine response when present at concentrations of 10^{-5} and 10^{-4} M. 10^{-3} M procaine usually causes an increase in amplitude of beat and at this concentration it is about as effective as tropacocaine.

Group C. Pentamethonium and Decamethonium.—Since the early reports concerning these bis-trimethylammonium polymethylene compounds (Barlow and Ing, 1948 ; Paton and Zaimis, 1948) there has been much interest in their mode of action (Paton, 1951 ; de Beer *et al.*, 1951). Decamethonium (C10) blocks at neuromuscular junctions in the vertebrates, whereas pentamethonium (C5) and the adjacent member of the series, hexamethonium (C6), block transmission at synapses in autonomic ganglia. Since we had concluded earlier that the general pharmacological pattern of the Venus heart resembled rather closely that of vertebrate autonomic ganglia, we assumed that C5

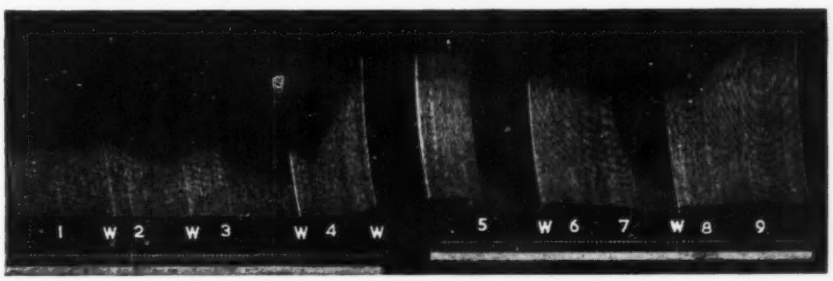

FIG. 2.—Record of the action of cocaine hydrochloride on the isolated ventricle of *Venus mercenaria*. 1 = 10^{-6} M cocaine, 2 = 10^{-5} M cocaine, 3 = 10^{-4} M cocaine, 4 = 10^{-3} M cocaine, 5 = 3×10^{-8} M acetylcholine, 6 = 10^{-4} M cocaine, 7 = 3×10^{-8} M acetylcholine, 8 = 10^{-3} M cocaine, 9 = 3×10^{-8} M acetylcholine. At W the drum was stopped and the bath flushed several times. Smallest time interval = 10 sec.

might antagonize the action of acetylcholine on the Venus heart. This proved not to be true. In fact, it was found that C5 showed no measurable effect until a concentration of 10^{-3} M was reached, at which it usually had an acetylcholine-like action and potentiated slightly the action of acetylcholine (Fig. 3A). On the other hand, C10 had generally a weak blocking action at 10^{-3} M (Fig. 3B). Like C5, it sometimes had a weak acetylcholine-like action in relatively high concentrations. With some hearts the actions of C5 and C10 were practically indistinguishable.

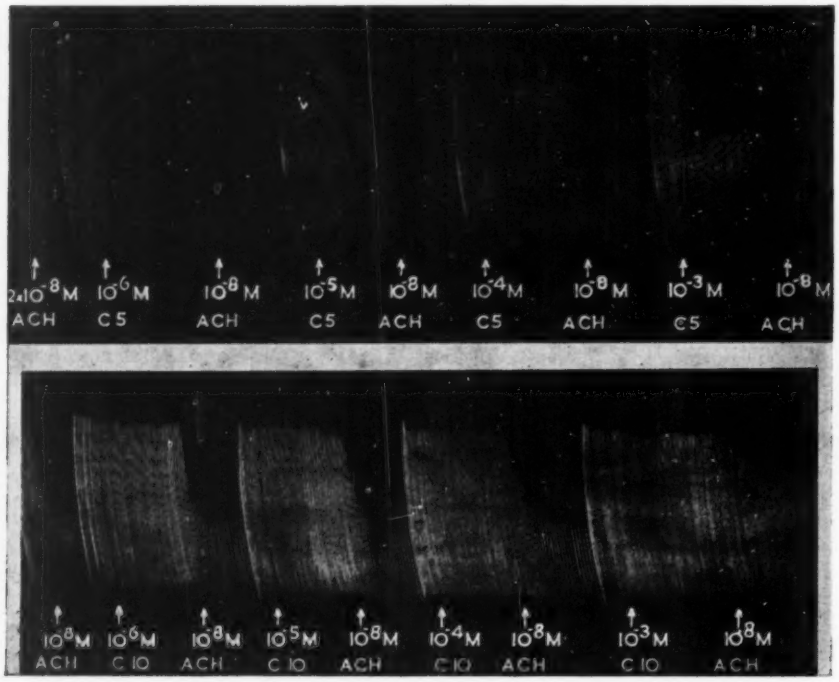

FIG. 3.—In A is shown a record of the actions of pentamethonium bromide (C5) and acetylcholine (Ach) on the isolated ventricle of *Venus mercenaria*. Note that 2×10^{-8} M acetylcholine was first used, but subsequently it was lowered to 10^{-8} M. In B is seen the effect of decamethonium bromide (C10) and acetylcholine.

It would appear that C5 and C10 both have weak acetylcholine-like actions on the Venus heart which may be attributed to the presence of $(CH_3)_3N^+$-groups in each (cf. Welsh and Taub, 1950), but that the 10 carbon chain in decamethonium made this compound a slightly effective antagonist at 10^{-3} M. Luduena and Brown (1952) found decamethonium to be inactive on the Venus heart at a concentration of 2.39×10^{-4} M.

Group D. Octyl- and Phenyl-trimethylammonium Compounds.—In this group are three trimethylammonium compounds and one dimethylethyl compound whose activities as potential acetylcholine antagonists are to be compared with the compounds in Group E. The first of these, *n*-octyltrimethylammonium, was found to have a weak acetylcholine-like action or to potentiate acetylcholine at 10^{-5} and 10^{-4} M concentrations, while at 10^{-3} M it had a moderate blocking action. Phenyltrimethylammonium iodide proved to be quite an active antagonist at all three of these concentrations. At 10^{-4} and 10^{-3} M it also had some acetylcholine-like activity.

The anticurare actions of 3-hydroxyphenyltrimethylammonium bromide (RO 2-2561), 3-hydroxyphenyldimethylethylammonium chloride (RO 2-3198 or "Tensilon"), and related phenolic salts were reported by Randall and Lehmann (1950), Randall (1950), and Wescoe and Riker (1951). It was of interest to find that RO 2-2561 and RO 2-3198 each had high acetylcholine-like activity on the Venus heart. Except in the case of 10^{-5} M tensilon it was not possible to determine the potential acetylcholine antagonism in the same manner as was done for the other compounds, hence the values appearing in brackets in Table I give the per cent decrease in amplitude of heart beat produced by these compounds.

A comparison of the activity of the phenyltrimethylammonium compound with that of the 3-hydroxyphenyltrimethylammonium shows that the latter has a more powerful acetylcholine-like action. This could be due to differences in solubility and adsorptive properties of these two substances. Randall (1950) observed that anticurare activity was reduced by a factor of five when the OH group of RO 2-2561 was shifted to the 2- or 4-positions, therefore the OH group in the 3-position appears to give a configurational superiority to the molecule in terms of anticurare action.

Group E. Di- and Tri-ethylammonium Compounds.—We had earlier noted the acetylcholine blocking action on the Venus heart of quaternary

ammonium compounds with three or four alkyl groups such as ethyl or *n*-propyl. The averaged data of Table II in Welsh and Taub (1950) can be recalculated to express the acetylcholine blocking action of 10^{-4} M concentrations of several *n*-alkyltriethylammonium compounds so that they may be compared with the compounds of Group E, Table I of this paper. Thus the blocking action of 10^{-4} M methyltriethylammonium salt is found to be 75% ; *n*-propyltriethylammonium, 78% ; *n*-amyltriethylammonium, 55% ; acetoxyethyltriethylammonium, 50% ; *n*-octyltriethylammonium, 93%. An interesting point to note is that the acetoxyethyltriethylammonium analogue of acetylcholine was the least effective of these five compounds, suggesting that, other things remaining constant, some departure from the acetoxyethyl-grouping favours antagonistic action on the Venus heart.

Whereas the addition of the 3-hydroxy-group increased the acetylcholine-like activity of the phenyltrimethylammonium salt, it is interesting that no marked difference was found in the antagonistic actions of phenyl- and 3-hydroxyphenyltriethylammonium salts.

Mytolon.—After the work thus far reported had been completed and we had concluded that the compounds of Group E represented the most effective acetylcholine antagonists for the Venus heart, there appeared the paper by Luduena and Brown (1952). From the results of their study it was apparent that there were far more effective anti-acetylcholine compounds for the Venus heart than any we had thus far tested.

Certain amino- and quaternary ammonium alkylamino-benzoquinone derivatives were first shown to be active curare-like agents by Cavallito, Soria,

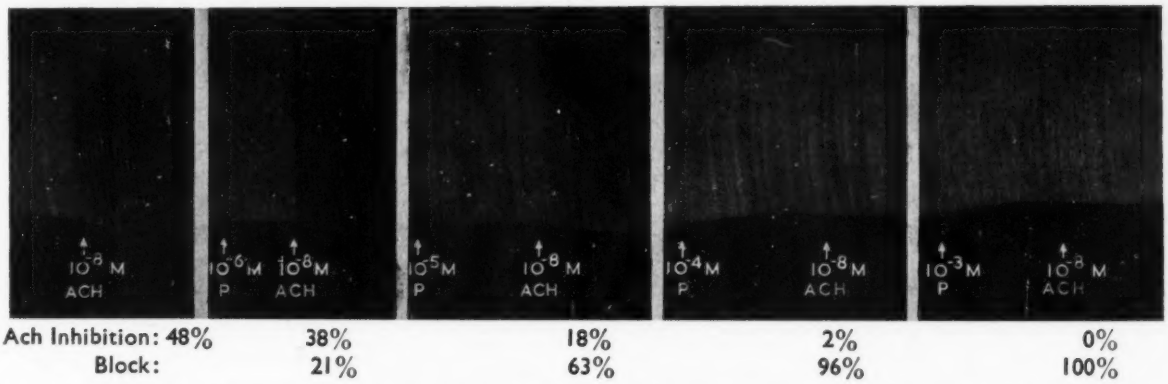

FIG. 4.—Record of the antagonism of the inhibitor action of acetylcholine (Ach) by phenyltriethylammonium bromide (P). Isolated ventricle of *Venus mercenaria*.

Banthine (β-diethylaminoethyl-9-xanthene carboxylate methobromide), a blocking agent at autonomic ganglia, was found to be less active, at the two lower concentrations, than the other compounds in Group E.

All four compounds markedly increased the amplitude of heart beat at one or more of the concentrations employed (Fig. 4). This was in contrast to the compounds of Group D. It was earlier suggested that this results from the blocking of endogenous acetylcholine, of which there is an appreciable amount in the heart. In order to obtain an estimate of the acetylcholine content of the Venus heart extractions and assays were made. Three determinations gave values equivalent to 0.47, 0.50, 0.7 μg. acetylcholine per gramme of wet weight of ventricle. For comparison the acetylcholine content of nerve ganglia of *Venus mercenaria* was found to be of the order of 5 μg./g. and that of adductor muscle 0.1 μg./g.

and Hoppe (1950). Of these compounds, the 2:5-bis(3′ - diethylaminopropylamino) - benzoquinone bis-benzyl chloride (WIN 2747 or mytolon chloride) was further studied by Hoppe (1950). This was shown to be the most effective curare-like member of the series. Luduena and Brown (1952) found mytolon an extremely active acetylcholine antagonist on the Venus heart. This is surprising, since it compares so favourably with decamethonium and *d*-tubocurarine as a neuromuscular blocking agent in vertebrates, yet neither of these has a significant acetylcholine-blocking action on the Venus heart except at a high concentration (10^{-3} M). We have now studied the effect of mytolon on the Venus heart and can affirm that it is in a distinctly different class from the other compounds we had earlier tested.

Using the same procedure employed with other agents, we find that 10^{-6} M mytolon completely blocks a dose of acetylcholine that would by itself

produce between 20 and 80% decrease in amplitude of heart beat. 10^{-8} M mytolon was found to block the action of such an amount of acetylcholine by about one-half.

Luduena and Brown had found that a concentration of 5.4×10^{-8} M mytolon reduced by 50% the inhibitory effect of a 5.5×10^{-8} M concentration of acetylcholine. They calculate that from two-thirds to three-fourths of the molecules of a given dose of acetylcholine are blocked by the same molar concentration of mytolon. We found, with different hearts, that the molecular blocking ratio of mytolon to acetylcholine varied from 2:1 to 5:1. We had earlier (Welsh and Taub, 1950) reported that from 1,500 to 3,000 molecules of tetraethylammonium were required to block one molecule of acetylcholine on the Venus heart. They found mytolon to be 980 times as effective as tetraethylammonium bromide on the Venus heart. Our quantitative data on mytolon, based on relatively few separate determinations, therefore agree very well with those of Luduena and Brown. They also found that maximal blocking activity required a benzyl and two ethyl groups on each of the nitrogens. The substitution of methyl for ethyl groups on the quaternary nitrogens (=WIN 3280) reduced the activity by one-third.

Discussion

The aim of this study was to acquire data from which we might make certain further deductions concerning the acetylcholine receptor substance of the Venus heart. We are still following the hypothesis put forth by one of us (Welsh, 1948) that acetylcholine receptor substances may be a family of enzymes for which acetylcholine serves as a coenzyme. While in many respects the pharmacology of the Venus heart resembles that of vertebrate autonomic ganglia it differs in some important respects. Most striking is the failure of pentamethonium to block acetylcholine on the Venus heart, whereas mytolon, which has little action at synapses in autonomic ganglia, is the most effective acetylcholine antagonist yet found for this organ.

We have shown earlier (Welsh and Taub, 1950, 1951) that the carbonyl group is an important combining group between acetylcholine and the receptor substance of the Venus heart. This was especially clear when the relative activities of 2-, 3-, and 4-ketoamyltrimethylammonium salts were determined. In the vertebrates these substances are primarily nicotine-like in their actions (Ing, Kordik, and Tudor Williams, 1952). Recently many workers (see Aeschlimann, editor, 1951), in accounting for the curare-like properties of a large number of new neuromuscular blocking agents, have placed much emphasis on the effectiveness of compounds containing two quaternary ammonium groups separated by a distance of approximately 14 Å. The belief, expressed or implied, is that this allows two ionic linkages to be formed between the nitrogens and correspondingly spaced acidic groups in the receptor molecule. A given number of bis-quaternary molecules could be more effective than twice as many corresponding half molecules, owing to the greater effectiveness of an anchor at both ends of the bis-compound.

It now seems logical to ask whether two or more carbonyl groups appropriately spaced in a molecule may not provide a double anchor keeping a molecule linked by more than one bond to points on a protein molecule where two molecules of acetylcholine might normally become attached. It has been suggested earlier in this paper that the acetylcholine blocking action of the barbiturates might be accounted for in this way. Decamethonium and *d*-tubocurarine are conspicuously poor acetylcholine antagonists on the Venus heart, as we have seen. In attempting to account for the far greater effectiveness of mytolon attention is drawn to the presence of two C=O groups in the benzoquinone ring, each of which might combine with a group in the receptor molecule. This would make possible two ionic linkages and two other bonds between mytolon and the receptor molecule of the Venus heart. No other feature would seem to account so adequately for the great superiority of mytolon as an antagonist to acetylcholine on the Venus heart. One might picture mytolon, and related compounds, lying more or less flat on the cell surface, attached by the two cationic groups (approx. 14 Å apart) to negatively charged groups in the receptor molecule and by the two carbonyl groups to approximately spaced OH- or NH_2-groups, as in semiquinone compounds.

Although this study adds only a small amount toward a fuller understanding of acetylcholine receptor substance, it confirms earlier views that the reaction between acetylcholine and cellular constituents is one in which molecular configuration is of great importance. The precise organization of groups (possibly amino-acid side-chains) within the receptor molecule may account in a large measure for the particular pharmacological pattern seen in a given organ or acetylcholine-sensitive region. Further attention should be paid to the possibility that such substances as the hydantoins and succinimides which have useful central nervous actions, as in the treatment of epilepsy,

are acting by blocking acetylcholine in the central nervous system.

Summary

1. The nature of the acetylcholine receptor substance of the heart of the lamellibranch mollusc, *Venus mercenaria*, was further explored by determining the relative activities of a number of acetylcholine antagonists.

2. Earlier work had indicated that the general pattern of response of this organ to acetylcholine analogues and to blocking agents was rather similar to that of vertebrate autonomic ganglia. Present results bring out important differences, e.g., the ineffectiveness of pentamethonium as an antagonist and the remarkable activity of " Mytolon."

3. The acetylcholine blocking action of certain substances having two carbonyl groups, such as the barbiturates and mytolon, leads to the suggestion that these groups may attach themselves simultaneously to the acetylcholine receptor molecule. This could account for the action of the large group of substances having two or more carbonyl groups that are useful as analgesics, hypnotics, and anticonvulsants.

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THE ROLE OF CHOLINESTERASE AT THE MYONEURAL JUNCTION

BY

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This paper describes the changes in the behaviour of the isolated rat phrenic nerve diaphragm preparation after its exposure to the action of inhibitors of cholinesterase, and attempts to correlate these changes with the variations in cholinesterase activity that may be taking place in the preparation. The values for residual cholinesterase activity have been calculated from data provided by the work of Davison (1953), who has studied the action of some inhibitors on the true and pseudo cholinesterase of the rat. He has also studied the speed with which the inhibition of these enzymes is reversed.

For most of the work the inhibitor used was *p*-nitrophenyl diethyl phosphate—paraoxon—which has been studied in some detail by Aldridge (1950) and Davison (1953). These observations on the isolated diaphragm show that function is transitorily disturbed while the activity of cholinesterase is in the process of reduction to about 10% of that normally present, and that it is impossible to abolish the response to indirect stimulation even under conditions where virtually no activity of cholinesterase would be expected to remain.

Burgen, Keele, and Slome (1949) found that tetra ethyl pyrophosphate (TEPP), an inhibitor with properties very similar to those of paraoxon, first enhanced the response of the isolated diaphragm to indirect stimulation, but later a marked decrease occurred. Lovatt Evans (1951) and Burgen and Hobbiger (1951) have studied the effect of a number of inhibitors on the ability of the diaphragm to sustain a response to a tetanus and the times taken for this effect to be reversed after the inhibitors have been removed. The observations recorded here are substantially in agreement with those earlier reports.

METHODS

The preparation of the rat phrenic-nerve diaphragm was essentially that described by Bülbring (1946). A special holder was designed, and the preparation works successfully when the muscle and nerve are completely submerged in the bath.

The holder (Fig. 1) is made of perspex. A $\frac{1}{4}$-in. brass rod is threaded into a perspex block. This block is cemented to the back of the top plate of a $\frac{3}{16}$-in. perspex stem fitted at its foot with $\frac{1}{8}$-in. diam. perspex rod which forms the anchor for attaching the costal margin of the half diaphragm. This stem also carries a wire to the diaphragm anchorage which can be used if required to stimulate the muscle directly, the current returning to earth via the stainless steel ligature used to attach the diaphragm to the lever assembly.

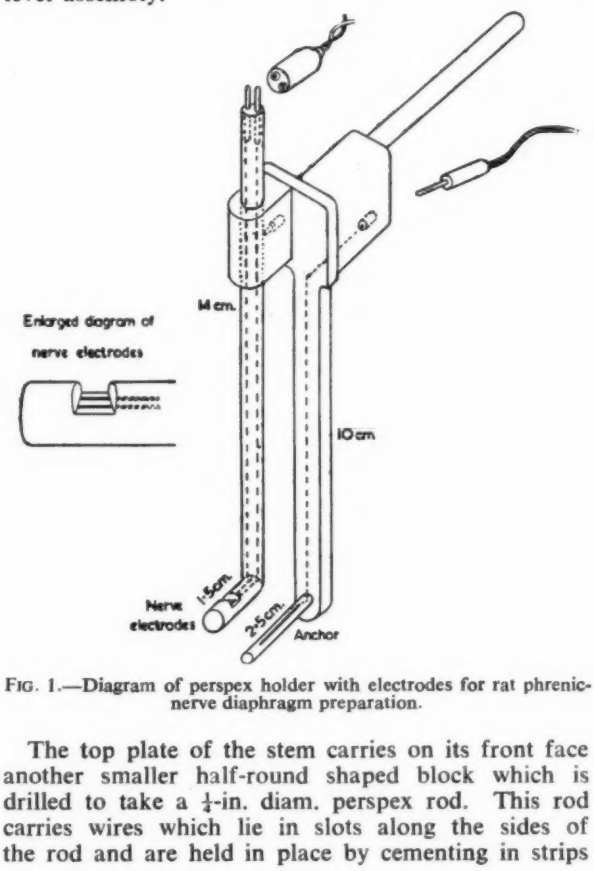

FIG. 1.—Diagram of perspex holder with electrodes for rat phrenic-nerve diaphragm preparation.

The top plate of the stem carries on its front face another smaller half-round shaped block which is drilled to take a $\frac{1}{4}$-in. diam. perspex rod. This rod carries wires which lie in slots along the sides of the rod and are held in place by cementing in strips

of perspex. These wires are four-stranded 39 S.W.G. stainless steel wires which are joined at the foot of the rod into short lengths of platinum wires which make contact with the nerve. A right-angled foot is made and a half section about 2 mm. is cut out forming a trough in which the nerve is laid and held in place by a rubber wedge. The platinum wires are laid across level with the floor of the trough. The rod carrying the nerve is adjustable vertically and can be rotated in its holder. The holder will eventually wear loose, and it is advisable to drill the holder block and insert a small spring-loaded plunger which bears gently on the rod, holding it in any required position.

The nerve was stimulated by rectangular impulses of 200 micro seconds duration and supramaximal intensity. They were normally applied at the rate of 8 per minute. Tetani at 50 per second were applied for 5 seconds.

The bath was 100-ml. capacity. Krebs solution at 37° C. aerated with $O_2+5\%$ CO_2 was used. Inhibitors of cholinesterase were added in ethanol in volumes of 0.1 ml. Acetylcholine was added in saline.

The names and inhibitory activity of the compounds used are given in Table I.

TABLE I

CHEMICAL NAMES AND ABBREVIATIONS OF THE INHIBITORS USED, TOGETHER WITH THEIR ACTIVITY AS INHIBITORS OF SHEEP RED CELL CHOLINESTERASE AND THEIR TOXICITY TO RATS

Chemical Name	Abbrev.	Molar Conc. Producing 50% Inhibition of Sheep RBC in 30 min.	Approx. Lethal Dose for Rats. mg./kg. Subcutaneously
Diethyl *p*-nitrophenyl phosphate	Paraoxon	$2{\cdot}1\times10^{-8}$	0·5
Tetraethyl pyrophosphate	TEPP	$1{\cdot}3\times10^{-8}$	0·3
Dimethyl *p*-nitrophenyl phosphate	Methyl paraoxon	$2{\cdot}5\times10^{-8}$	0·5
Di-isopropyl *p*-nitrophenyl phosphate	Isopropyl paraoxon	$4{\cdot}7\times10^{-7}$	5·0
Di-isopropyl fluorophosphonate	DFP	$4{\cdot}6\times10^{-7}$	2·0

RESULTS

The Effect of Adding Paraoxon to the Isolated Diaphragm

Stimulation at 8 per Minute.—If paraoxon is added to give a final concentration of 4×10^{-7} M in the bath containing the diaphragm there is a latent period of 2 minutes before the contractions begin to increase in size ; they then rapidly become from 50–200% greater in extent than the original ones.

The contractions remain at their maximum for only one or two responses and then rapidly diminish and return to their original size all within 10 minutes of adding the inhibitor (Fig. 2). During the period of enhanced contractions, fasciculations and irregular twitchings of the diaphragm can be seen, but these diminish as the contractions return to their original size.

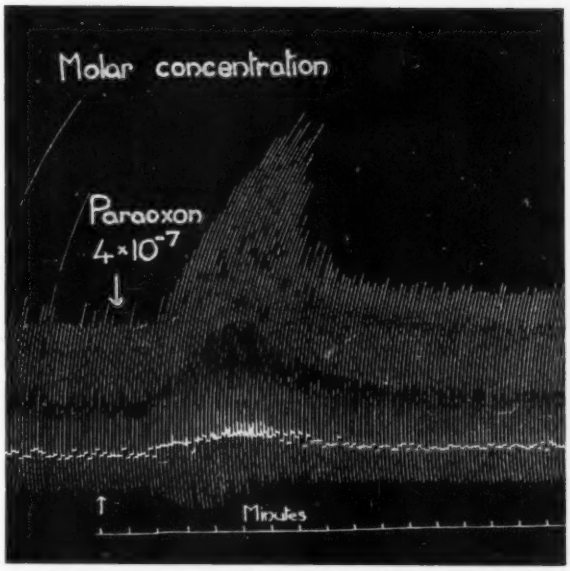

FIG. 2.—Rat phrenic-nerve diaphragm. Indirect stimulation 8 per min. Paraoxon (4×10^{-7}M) added. Marks of irregular twitchings can be seen at base line during the period of enhanced contractions.

After returning to its original level the response remains unchanged even though the diaphragm is left in the solution of inhibitor for several hours. Under these conditions no appreciable hydrolysis of paraoxon would take place.

Tetanus at 50 per Second for 5 Seconds.—The normal diaphragm responds by a sustained contraction. When paraoxon is added the ability to hold this response is lost during the period of the enhanced contractions and does not return as long as the inhibitor remains in the bath.

Sensitivity to Acetylcholine.—The contractions of a normal diaphragm stimulated at 8 per minute are reduced by 50% if acetylcholine is added to give a final concentration of $2–3\times10^{-3}$ M, and they are abolished at 5×10^{-3} M. On washing out the acetylcholine, the responses immediately return. They may be reduced or abolished by acetylcholine for periods of at least an hour, but will immediately return when it is removed.

After the addition of paraoxon (4×10^{-7} M) for 30 minutes the sensitivity of the diaphragm increases so that the response is now reduced by 50% in 5×10^{-6} M acetylcholine and abolished in 5×10^{-5} M (Fig. 3).

If acetylcholine is added to a suspension of red cells, it will protect the cholinesterase from inhi-

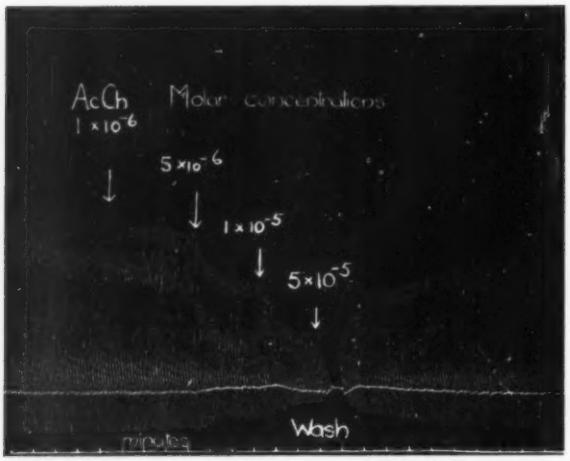

FIG. 3.—Rat phrenic-nerve diaphragm. Indirect stimulation 8 per min. Showing the effect of acetylcholine at different concentrations on size of response after previous incubation with 4×10^{-7} M paraoxon.

bition by paraoxon added subsequently. A concentration of 1×10^{-2} acetylcholine added to the diaphragm abolishes its response to stimulation and will also protect it from the full action of 1×10^{-7} M paraoxon, for, if both acetylcholine and inhibitor are removed after 1 hour, the diaphragm still retains its power to hold a tetanus. A concentration of 5×10^{-3} M acetylcholine will abolish the response of the diaphragm to stimulation, but will not protect it from the action of 4×10^{-7} M paraoxon, for the power to sustain a tetanus is lost when tested after removal of acetylcholine and inhibitor.

Effect of the Removal of Paraoxon.—In many preparations the removal of paraoxon by 3 washings after ½–1 hour had no effect on the size of the contractions at 8 per minute, but in a few there was some increase (10–20%). No other change was observed in periods of observation of up to 3 hours. The response to a tetanus, on the other hand, gradually changed, so that 30–40 minutes after removal of the inhibitor the preparation had regained its ability to hold a tetanus for 5 seconds. Over the next 3 hours the preparation gradually lost some of its sensitivity to acetylcholine, so that at the end of 3 hours a concentration of 1×10^{-4} M produced only a 33% reduction in the size of the contraction.

Effect of a Second Dose of Paraoxon.—The addition of paraoxon (4×10^{-7} M) one hour after the first dose had been removed had no effect on the response to regular stimuli. There was no enhancement, and no fasciculations or twitchings were seen. On the other hand, the ability to hold a tetanus was lost and the sensitivity to acetylcholine again increased. These changes were reversed by removing the inhibitor. This procedure has been repeated on as many as five separate occasions on one preparation in a day. The enhanced response was seen only after the first dose of paraoxon.

Effect of the Concentration of Added Paraoxon.—The changes in the response of the diaphragm to paraoxon and their reversal are presumably attributable to the progressive inhibition of cholinesterase and the reversal of this effect after removal of the inhibitor. The speed with which the changes in the response to the first dose of paraoxon take place depends upon the concentration of the inhibitor that is added, and some comparative figures are given in Table II.

TABLE II

THE EFFECT OF CONCENTRATION OF PARAOXON ON THE SPEED WITH WHICH CHANGES TAKE PLACE IN THE ISOLATED DIAPHRAGM

Concentration of Paraoxon, M.	Time (min.) between the Addition of the Inhibitor to the Bath and:		
	(*a*) 1st Enhanced Contraction	(*b*) Loss of Tetanus	(*c*) End of Enhancement
4×10^{-8}	13	39	
1×10^{-7}	6	18	20
4×10^{-7}	2	8	8
4×10^{-6}	0·5	1	1·75

In other experiments the response to tetanus was observed at 2-minute intervals from the time of adding the inhibitor until there was a complete failure to hold a sustained response. At concentrations of 2×10^{-8} M up to 1×10^{-7} M paraoxon this period was proportional to the concentration of inhibitor, but at concentrations of from 2 to 8 $\times 10^{-7}$ M the time taken for failure was not proportional to the concentration, and it appeared to take longer for the inhibitor to exert its effect. At concentrations greater than 8×10^{-7} M the interval was too short for measurement by this method. The disproportion between the concentration of inhibitor and the time taken for the production of an effect when this interval becomes very short may possibly be due to the time needed for the inhibitor to penetrate the diaphragm. This time only becomes significant when the action of the inhibitor is rapid.

The time taken for the reversal of one of the effects of the inhibitor, namely the return of the ability to hold tetanus, was also measured in some preparations. This was independent of the time during which the inhibitor had been in contact with the diaphragm, but was greater after exposure to higher concentrations of paraoxon.

If the return of function is due to a reversal of inhibited cholinesterase the time taken should be independent of the concentration of inhibitor used. An explanation of the discrepancy could be that additional time was needed for the high concentrations of inhibitor to diffuse out of the diaphragm into the bath.

It was important to find out whether paraoxon reacts with the cholinesterase in the rat's diaphragm in the same way that it does with other preparations of cholinesterase.

Diaphragms were exposed for 30 minutes to 2×10^{-8} M paraoxon in the bath. This was calculated to give 58% and 41% inhibition respectively of true and pseudo cholinesterase. After 30 minutes the diaphragms were removed, washed and homogenized, and the activity of the true and pseudo cholinesterase determined manometrically. Half of each diaphragm was not incubated in paraoxon and served as a control. In two experiments the activity of true cholinesterase had been inhibited by 53% and 57% (theoretical 58%) and that of pseudo cholinesterase inhibited by 31% and 53% (theoretical 41%). It therefore appears that inhibition of the cholinesterase of the rat diaphragm by paraoxon occurs at similar rates to the inhibition of cholinesterase in red cells or tissue homogenates.

The effect of increasing the concentration of paraoxon was to increase the speed with which the changes in the response of the diaphragm took place. If the concentration of paraoxon was increased to 4×10^{-4} M the preparation continued to respond quite satisfactorily to stimulation at 8 per minute. A preparation of red cell cholinesterase would be completely inhibited within a few seconds of the addition of paraoxon at this concentration.

Reaction of the Diaphragm to Other Inhibitors

As most of the work with paraoxon had been done in a concentration of 4×10^{-7} M, other inhibitors were added in a concentration that would give a comparable degree of inhibition. These figures were derived from the data in Table I on the activity against red cell cholinesterase.

With TEPP (2×10^{-7} M) the reactions of the diaphragm and pattern of reversal were indistinguishable from those with paraoxon.

With DFP (1×10^{-5} M) and isopropyl paraoxon (1×10^{-6} M) the initial response of the diaphragm was the same as with paraoxon, and the diaphragm continued to respond to regular stimuli for an indefinite period. On removing the inhibitor, however, there was no return of the power to hold a tetanus within a period of 4 hours. Sensitivity to acetylcholine diminished slightly after the inhibitor had been removed, but did not continue to do so over the next 3 hours.

After methyl paraoxon (1×10^{-6} M) the responses to the addition of the inhibitor are the same, but on removing it reversal occurs very rapidly and the ability to sustain a tetanus is regained within 3 minutes. If a second dose of methyl paraoxon is given 45–60 minutes after removing the first dose there is a period of enhanced contractions similar to that seen after the first dose. Aldridge (1953) has shown that methyl paraoxon produces an inhibition of true cholinesterase that is rapidly reversed.

Attempt to Obtain Complete Inhibition of Cholinesterase

Although very high concentrations of paraoxon (4×10^{-4} M) should produce virtually complete inhibition of cholinesterase there is an appreciable rate of reversal of cholinesterase inhibited by paraoxon. This means that an equilibrium must finally be established between inhibition and reversal at some stage before inhibition is absolutely complete. The level of cholinesterase activity at which such an equilibrium must finally be established could be further reduced by choosing an inhibitor from whose effects reversal was slower than paraoxon. A comparison was therefore made between the performance of diaphragms exposed to paraoxon (4×10^{-4} M) and to isopropyl paraoxon (1×10^{-4} M) for 1 hour. The preparations were stimulated for $\frac{1}{2}$ minute at different rates. Both gave a normal response to stimuli at 8, 16, 30, and 60 per minute. The diaphragm in paraoxon responded normally to stimuli at 2 and 5 per second, but that in isopropyl paraoxon failed to do so. At 10 per second the paraoxon diaphragm also failed. Thus under conditions where very small differences in residual cholinesterase activity must be present a difference in the performance of the preparation could be detected.

The Behaviour of Diaphragms Taken from Poisoned Rats

Rats were killed by the subcutaneous injection of 2 mg./kg. paraoxon. Death took place in 7–15 minutes and the chest was opened immediately and the diaphragm removed.

It took 15–60 minutes from the time of death to the first recording of a response of the diaphragm in the bath. Under these conditions the diaphragm would respond to single stimuli but failed to sustain a tetanus. The ability to hold a tetanus

returned gradually and was complete within 30–60 minutes. The diaphragm from a rat killed with isopropyl paraoxon showed no recovery of the ability to hold a tetanus.

Discussion

The addition of an inhibitor of cholinesterase to the isolated diaphragm produces a succession of changes to its response to indirect stimulation. The speed with which the changes take place is proportional to the concentration of inhibitor added, suggesting that they accompany the gradual changes in the activity of the cholinesterase in the preparation. Davison (1953) and Denz (1953) have shown that the diaphragm of the rat contains both true and pseudo cholinesterase, and it was found that the inhibition of these enzymes in the isolated preparation exposed to paraoxon occurred at a rate similar to that observed on other rat cholinesterase preparations.

Davison (1953) has been able to derive rate constants for the inhibition of both true and pseudo cholinesterase of the rat when acted on by paraoxon. It therefore seemed permissible to use these figures in order to calculate how much activity could be expected to remain in the diaphragm at different times after exposure.

The calculated rate constants for inhibition by paraoxon are as follows (Davison):

Rat true ChE .. 1.44×10^6 (min.$^{-1}$)
Rat pseudo ChE .. 8.7×10^5 (min.$^{-1}$)

and from these the activity of cholinesterase that would remain in the diaphragm at different times after adding the inhibitor (1×10^{-7} M paraoxon) can be calculated, and is as follows:

Time after Adding Paraoxon (min.)	Condition of Diaphragm	Calculated Activity %	
		True	Pseudo
6	Beginning of enhanced contraction	42	60
18	Failure of tetanus	8	22
20	End of enhanced contraction ..	5	17
60	50% inhibition by 5×10^{-6}M acetylcholine	0·1	0·5

It will be seen that when about half of the activity of cholinesterase is destroyed the response of the diaphragm to single stimuli increases and there is a period of fasciculation and irregular twitching, but as the inhibition progresses and nears completion these effects disappear, but the ability to hold a tetanus now fails. But this does not occur until less than 10% of the cholinesterase remains. However, a normal response to indirect single stimuli of 200 m.sec. duration is obtained after 1 hour in 1×10^{-7} M paraoxon when the cholinesterase activity must have become reduced to extremely low levels. Even a thousandfold increase in the concentration of inhibitor or the use of an inhibitor which produces an apparently completely irreversible inhibition (isopropyl paraoxon) will not abolish this response.

Davison has also determined the rate constants for the reversal of true and pseudo cholinesterase of the rat inhibited by paraoxon. His figures are as follows:

Reversal of true ChE after paraoxon ... 8×10^{-5} (min.$^{-1}$)
Reversal of pseudo ChE after paraoxon 2×10^{-3} (min.$^{-1}$)

The activity of true and pseudo cholinesterase that will have returned in the diaphragm after exposure to paraoxon may therefore be calculated, and is as follows:

Time after Removing Paraoxon (min.)	Condition of Diaphragm	Calculated Return of Activity %	
		True	Pseudo
30	Able to hold tetanus	—	3
60	50% inhibition by 6×10^{-5}M ACh	—	11
120	$1{\cdot}2 \times 10^{-4}$M ACh	—	21
180	2×10^{-4}M ACh	1·5	30

If the reversal of cholinesterase takes place at the same rate in the isolated whole diaphragm as it does in other cholinesterase preparations *in vitro*, it will be seen that very little activity is needed in order to allow the diaphragm to regain its ability to show a sustained response to a tetanus. There is a big difference in the speeds with which pseudo and true cholinesterase reverse after inhibition by paraoxon, and these calculations suggest either that pseudo cholinesterase might be able to carry out some function at the myoneural junction, or that only extremely small quantities of true cholinesterase are necessary. The possibility cannot be excluded that in the intact diaphragm the reversal of the inhibited enzymes is catalysed by some unknown factor not present in the homogenized tissue preparations from a study of which the rate constants for reversal were derived.

Interest seems to centre on two features of these observations. It is impossible to prevent conduction across the myoneural junction by inhibitors of cholinesterase. There is good evidence that they can reach the bulk of the cholinesterase, and histochemical observations show that in their presence in concentrations such as those used here no staining at all of cholinesterase takes place at the

myoneural junction (Denz, 1953). The conclusion therefore must be either that cholinesterase is not essential for the functioning of the myoneural junction under these conditions, or that some very small fraction of the enzyme at the myoneural junction is protected from the action of the inhibitor.

The second point of interest is that the maximum derangement of response of the muscle to indirect stimulation takes place when between 50% and 80% of the cholinesterase is inactivated, but after that the preparation again responds in an orderly fashion to single stimuli.

Furthermore, on removal of an inhibitor such as paraoxon which produces an inhibition that is only slowly reversible, the ability of the preparation to respond to tetanus is restored, but the fasciculations and enhanced response to single stimuli do not reappear. If an inhibitor producing a more rapidly reversible inhibition of cholinesterase (e.g., methyl paraoxon) is used, the addition of a second dose of inhibitor does result in enhanced responses.

A similar picture is seen in the whole animal poisoned with paraoxon and revived from the effects of a lethal dose by artificial respiration. The widespread fasciculations disappear and natural respiration returns, but the animal is not seriously disturbed by a second "lethal" dose of the inhibitor (Barnes, 1953).

The diaphragms from rats killed by paraoxon cannot sustain a tetanus when first set up in the bath, but it seems probable that failure of myoneural conduction is not responsible for the death of animals after TEPP (Douglas and Matthews, 1952). In any case such a diaphragm will still respond satisfactorily to single stimuli.

It seems possible that the widespread disturbances during the reduction of the cholinesterase if they occur in tissues other than muscle (e.g., the brain) may be responsible for death, but that, if this is averted by artificial respiration, then a more orderly function is restored once the level of cholinesterase has been reduced almost to zero. At this stage further doses of anticholinesterase will not interfere with function.

Summary

1. The action of inhibitors of cholinesterase upon the isolated rat phrenic-nerve diaphragm preparation has been studied.

2. The response to indirect stimulation goes through a series of changes as the enzyme is inhibited, but settles down to an apparently normal response once inhibition is complete.

3. It is impossible to prevent the response to single stimuli even by very high concentrations of inhibitor, but the response to a tetanus fails.

4. Both true and pseudo cholinesterase are present in the rat diaphragm, and they respond to inhibitors in the same way as cholinesterase in other tissues.

5. Calculations have been made which show that fasciculations and enhanced response to single stimuli take place while the cholinesterase activity is reduced from 50% down to 10% of normal, but then disappear.

6. The power to sustain a tetanus disappears when only about 10% of cholinesterase remains, but when reversal from complete inhibition is allowed to occur by removing the inhibitor, the power returns when calculations suggest that less than 5% of the pseudo and virtually no true cholinesterase has had time to be released from inhibition.

We wish to thank Mr. A. N. Davison for carrying out the cholinesterase determinations on the diaphragms exposed to paraoxon. Mr. C. R. Kennedy and Mr. H. McKinnon were responsible for the design and construction of the holder for the diaphragms.

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OBSERVATIONS ON THE EFFECTS OF A SERIES OF DEXTRAN SULPHATES OF VARYING MOLECULAR WEIGHT ON THE FORMED ELEMENTS OF THE BLOOD *IN VITRO*

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The sulphuric esters of dextran, a complex polymer of glucose, show heparin-like anticoagulant activity (Grönwall, Ingelman, and Mosimann, 1945; Ingelman, 1947; Ricketts, 1952). In a previous communication (Walton, 1952) it was reported that differences of behaviour were demonstrable between members of a series of dextran sulphates prepared from parent dextrans of varying molecular weight. Although all the compounds behaved qualitatively like heparin in showing antithrombin activity, it was found that the compounds with molecular weights above a certain critical level caused, in addition, the precipitation from solution of fibrinogen (and possibly other macromolecular proteins) when mixed with plasma under physiological conditions. On the other hand, under similar conditions, the members of the series of dextran sulphates with molecular weights below this critical level did not interfere with the stability in solution of the plasma proteins.

It was found that the compounds which caused precipitation of the plasma-proteins also caused agglutination of the white-cells and blood platelets and produced alterations of the erythrocyte sedimentation rate. The compounds which did not precipitate the plasma-proteins produced no demonstrable alteration in the formed elements of the blood. The present communication presents details of the investigation of these differences in the reactions of the various blood cells to the dextran sulphates of large and of small molecular weight. The effect of various heparin samples on the formed elements of the blood was investigated simultaneously.

MATERIALS AND METHODS

Dextran Sulphates.—The compounds employed were selected members of the series previously described (Ricketts, 1952; Walton, 1952). They formed two main molecular weight groups:

Large Molecular Weight Group.—The compounds originally examined in this group ranged in molecular weight from 515,000 to 39,800. They were divided into three sub-groups: D (3 samples), of average molecular weight 458,000; A (2 samples), of average molecular weight 129,000; and E (2 samples), of average molecular weight 47,000. The samples within each sub-group originated from the same parent dextran, but differed in the extent of their sulphation. It was found that, in relation to their effects upon the blood cells, the compounds in any one sub-group produced qualitatively similar effects. For the sake of brevity, only the results obtained with the compounds of sub-groups D and E are reported. The middle sub-group, A, produced intermediate effects.

Small Molecular Weight Group.—This contained only one sub-group, I (3 samples), of average molecular weight 7,500.

Heparin.—Commercial heparin preparations were used for qualitative comparisons with the dextran sulphates. For most purposes, the preparation used was "Pularin" (Evans), batches No. F85020 and No. N10090, both assaying at 90 i.u. per mg. For more extended investigation of the effect of heparin upon platelets, the following additional preparations were employed: Heparin (Boots), batch No. 3583, 102 i.u. per mg.; "Liquemin" (Roche), batch dated 31.8.48, 122 i.u. per mg.; Heparin (British Drug Houses), batch No. 30607/c, 160 i.u. per mg.

Siliconed Glass-ware.—Two silicone preparations were employed: (1) "Teddol" (British Thomson-Houston Co. Ltd.), which is stated to be a mixture of methyl-chlorosilane and dimethyl-chlorosilane. A 1% solution in petroleum ether was applied repeatedly to the glass-ware, which was drained and allowed to dry in a fume-cupboard between applications. The vessels so treated were washed well with distilled water before use. (2) "DC 1107" (Albright and Wilson Ltd.) as a 3% solution in carbon tetrachloride was applied to the glass-ware, which was then drained and subsequently baked in an oven at 150° C. for fifteen minutes.

Both compounds were found to be effective in prolonging the coagulation time of the blood and in

preserving the platelets, but it was found to be essential to use perfectly clean, unscratched glass-ware and to ensure complete coating of the treated surfaces by repeated applications to obtain results comparable with those previously reported (Jaques, Fidlar, Felstead, and MacDonald, 1946; Patton, Ware, and Seegers, 1948).

Platelet-counting Methods.—Lempert's (1935) modification of Kristenson's (1922) method, and the method of Rees and Ecker (1923), were used. The latter method was modified by the substitution of 1% formalin for the 0.1% concentration originally suggested.

At high concentrations of heparin and of the dextran sulphates, it was found that the anticoagulants formed insoluble precipitates with the brilliant cresyl blue contained in both diluting fluids. For this reason, in some experiments, the dye was omitted and counting performed with the phase-contrast microscope.

Toluidine Blue.—Batch No. 851 of toluidine blue G (G. T. Gurr Ltd.) was used as a 1% (w/v) solution in distilled water.

Results

Effect of Heparin and Dextran Sulphates on Whole Blood in vitro

It was reported previously (Walton, 1952) that the D and E size (large molecular weight) dextran sulphates caused precipitation of fibrinogen on addition to plasma or fibrinogen solutions. The precipitates formed, under physiological conditions of temperature, electrolyte concentration, etc., were found to be loose, easily dissociable complexes. Their formation was shown to be dependent primarily upon the molecular size rather than the content of sulphate groups of the acid polysaccharides. For this and other reasons the reaction was regarded as being due mainly to the interplay of non-ionic forces between the similarly-shaped asymmetrical molecules of the reactants. The effect of this interaction upon the stability of the formed elements of the blood was investigated in the following manner:

Experiment 1.—3 mg. each of heparin and of the D, E, and I size dextran sulphates was weighed out in siliconed glass tubes. A fifth tube contained the oxalate mixture of Heller and Paul (1934). 20 ml. of blood was withdrawn from the antecubital vein of a normal human subject, using a siliconed syringe and needle and with the adoption of the usual precautions to avoid contamination with tissue-juices. The first and last few ml. of blood in the syringe was discarded and the remainder was divided between the tubes containing the anticoagulants so that each tube contained 3 ml. of blood. The tubes were rotated gently to ensure solution of the anticoagulants in the blood. Thin smears (as for a differential white cell count) were prepared from the blood in each tube. Platelet-counts were then performed on each blood sample. Following this, the tubes were allowed to stand undisturbed for two hours, but were observed from time to time during this period. These observations showed that the rate and the final extent of sedimentation of the red-cells was approximately the same in the tubes containing oxalate, heparin, and the I size dextran sulphate, but was markedly less in the tubes containing the large molecular weight dextran sulphates. It was also noted that the plasma in the heparin, oxalate, and small molecular weight dextran sulphate tubes showed the shimmering opacity which is observed normally, while the plasma in the tubes containing the D and E size dextran sulphates was quite clear and transparent. Platelet-counts were now carried out on the plasma in each tube. The results are shown in Table I, from which it can be seen that there was good agreement between the platelet-counts in whole blood and in plasma treated with oxalate, heparin, and the I size dextran sulphate. On the other hand, the counts in the whole blood treated with the D and E size dextran sulphates were reduced while the counts in the corresponding plasmas (after sedimentation of the red-cells) were even more markedly diminished.

When the blood-smears made at the beginning of the experiment were stained with Leishman's or Giemsa's stain and examined microscopically, large basophilic masses were noted near the edges and in the "tails" of the smears made from the blood treated with the D and E size dextran sulphates. At the periphery of these masses individual platelets could be discerned, while the centres consisted of amorphous material in which large numbers of agglutinated platelets and occasional polymorph leucocytes were enmeshed (see Fig. 1).

Examination of the rest of the smear showed only an occasional discrete platelet here and there. Smears

TABLE I

EFFECT OF HEPARIN AND OF THE DIFFERENT DEXTRAN SULPHATES UPON PLATELETS IN WHOLE BLOOD *IN VITRO*, AS COMPARED WITH OXALATE

Compound	Platelet-Count*		Appearance of Blood-smear	Haematocrit (%)	Supernatant Plasma	Buffy Layer (%)
	Whole Blood	Plasma				
D size	36,000	< 10,000†	Basophilic clumps ++	38	Clear	2·0
E size	124,000	< 10,000†	" "	38	"	1·5
I size	336,000	310,000	Single platelets	38	Opalescent	1·0
Heparin	328,000	304,000	" "	38	"	1·0
Oxalate	352,000	308,000	" "	38	"	1·0

* Mean of three counts expressed as number per c.mm.
† Single platelets only counted. Many platelet clumps also present.

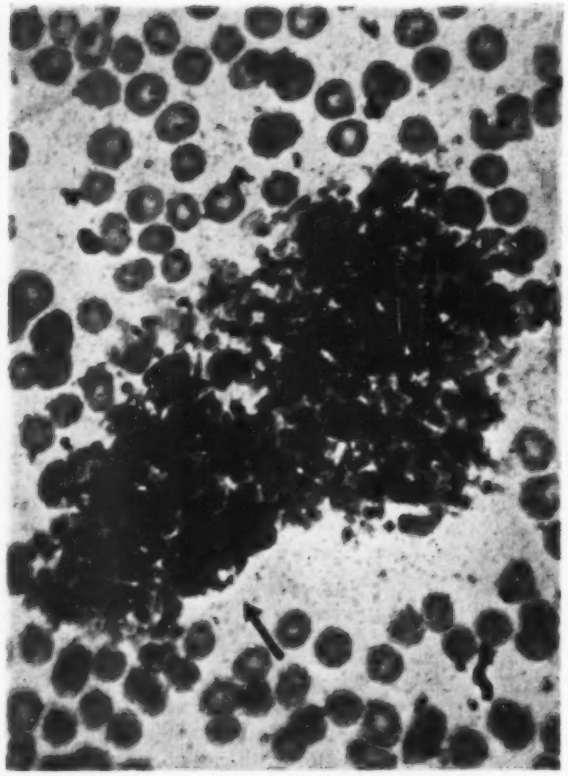

FIG. 1.—The effect of adding the D size dextran sulphate to whole blood *in vitro*. The blood-smear contains an irregular mass, consisting of a large number of platelets and a single leucocyte (marked by the arrow) clumped together. Leishman's stain. × 700.

made from the blood treated with oxalate, heparin, or the I size dextran sulphate showed the normal random distribution of single platelets with only occasional small platelet-clumps. These were free from basophilic material. No abnormal morphological changes were noted in the red or the unagglutinated white cells, in any of the smears.

Finally, the blood from each tube was transferred to a haematocrit tube and centrifuged at 3,000 r.p.m. for 30 minutes. As noted in Table I, the volume of packed red-cells in all the tubes was identical, but the tubes containing the large molecular weight dextran sulphates showed a thicker "buffy coat." When removed and examined microscopically, the buffy coat from the oxalate, heparin, and I size dextran sulphate tubes was found to consist of the usual mixture of large numbers of leucocytes and erythrocytes with a few small clumps of platelets and large numbers of single platelets. The buffy coat from the tubes containing the D and E size dextran sulphates consisted of leucocytes and erythrocytes in apparently normal numbers, but there were also present many shreds and loose flocculent masses of material to which platelets and leucocytes were adherent. Few discrete platelets were seen (see Fig. 2). When a drop of 0.1% brilliant cresyl blue was added to a hanging-drop preparation made from the buffy coat in the D or E size dextran sulphate tube, each flocculent mass was seen to consist of material staining a bright reddish-purple with large numbers of dark blue platelets clustered on the surface or interspersed among it.

From the results of this experiment, summarized in Table I, it appeared that the small molecular weight dextran sulphate, like heparin or oxalate, had had little or no effect on the formed elements of the blood. This variety of dextran sulphate had been shown previously not to affect the stability of the plasma-proteins. On the other hand, the large molecular weight dextran sulphates which had been found previously to form insoluble complexes with fibrinogen and other macromolecular plasma proteins, were now found to cause agglutination of platelets and white-cells. The behaviour of these compounds in relation to the individual formed elements of the blood was investigated further.

Effect of the Dextran Sulphates on Platelets

It was noted in Experiment 1 that in each platelet-clump found in the buffy coat of blood treated with the large molecular weight dextran sulphates,

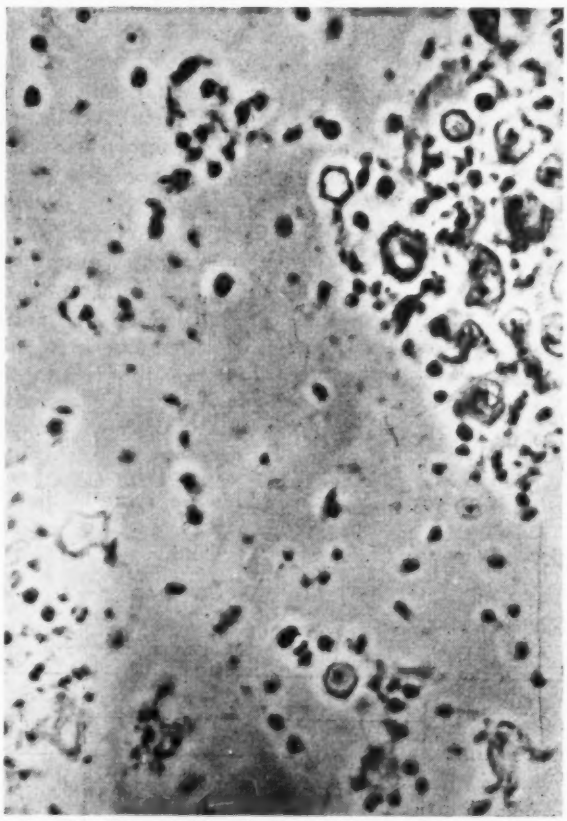

FIG. 2.—The effect of adding the E size dextran sulphate to plasma *in vitro*. A mass of agglutinated platelets and leucocytes is seen at the top right. Smaller clumps are present at the top centre and bottom right, while scattered single platelets are present elsewhere in the field. Unstained preparation photographed under the phase-contrast microscope. × 430.

the nidus of the clump consisted of amorphous material. This material was found to be strongly metachromatic when stained with cresyl blue. It was found that dextran sulphate alone or in combination with fibrinogen stained in this manner also. It therefore seemed reasonable to suppose that the clumping of the platelets occurred because of their adherence to the freshly formed surface of the complex or because the platelets acted as foci for the formation of the particles of the precipitated complex. In order to test these hypotheses the following experiment was undertaken:

Experiment 2.—A suspension of platelets in saline was prepared as follows: 20 ml. of blood was taken in a siliconed syringe from a normal human subject. The blood was placed in a cooled, siliconed glass tube and centrifuged at 0° C. and at 15,000 r.p.m. for 30 minutes. The plasma was removed and the buffy coat taken up in 2 ml. of 0.85% saline. This saline suspension was repeatedly centrifuged at 15,000 r.p.m. and washed in the cold with saline till a sample of the supernatant no longer gave a precipitate on the addition of a drop of 10% trichloracetic acid. The deposit was then judged to be free from plasma protein. It was resuspended in saline and examined under the microscope. It was found to consist of leucocytes, large numbers of single platelets, and many platelet-clumps. The suspension was then centrifuged lightly to remove the leucocytes and clumped platelets. The supernatant, consisting almost entirely of single platelets, was withdrawn. Five drops of this suspension were placed in each of four siliconed tubes, using a siliconed pipette. One drop of a 1% solution of heparin or of one of the dextran sulphates was then added to each tube. The tubes were shaken gently and one drop of each mixture in turn was placed on a siliconed microscope slide and examined under a phase-contrast microscope. In a second series of siliconed tubes, similar mixtures of the anticoagulants were made with the platelet suspension, but now to each tube there were added two drops of citrated plasma or serum. Again a drop of the final mixture from each tube was removed and examined. The contents of a control tube containing the platelet suspension mixed with plasma or serum alone were also examined.

It was found that the addition of heparin or of any one of the dextran sulphates to the saline suspension of platelets produced no alteration. But when serum or plasma was added to the mixture containing the large molecular weight dextran sulphates, the platelets could be seen to become entrained in, and adherent to, the precipitate which slowly formed, in a manner similar to that observed previously (see Fig. 2). The volume of precipitate and the amount of platelet-agglutination which occurred were both markedly greater with plasma than with serum. The addition of plasma or serum to the mixtures containing the I size dextran sulphate produced no precipitation of protein and the platelets remained unagglutinated.

In confirmation of the finding of Copley and Houlihan (1947) it was observed that certain samples of serum (whether homologous or heterologous in relation to the platelets) caused apparent agglutination of the saline suspension of platelets in the control tube. Such samples of serum were rejected. Further examination of these apparently spontaneously-formed clumps of platelets in certain samples of serum and plasma showed that gentle pressure on the cover-slip or microprobing with a very fine siliconed glass probe resulted in dispersal of the clump into its individual platelet elements. On the other hand, similar treatment of the much larger and more numerous clumps in serum or plasma containing the large molecular weight dextran sulphates did not result in similar dispersal. Instead, it was observed that, with the passage of time, the platelets present in the clumps slowly underwent granular disintegration so that the clump was eventually composed of amorphous material in which no separate elements were distinguishable. At a corresponding interval, the clumps formed in serum alone or in plasma containing citrate, oxalate, heparin, or the I size dextran sulphate were still composed of individually distinguishable platelets, and these could still be dispersed in the manner described above.

In other experiments various other particulate suspensions were substituted for the saline suspension of platelets. The materials used included a formolized suspension of *S. marcescens* in distilled water, watery suspensions of colloidal graphite varying in particle size from 0.5 μ to 3.0 μ, and a watery suspension of finely powdered kaolin (Crooke's collosal kaolin). In some experiments a 0.6% solution of human fibrinogen was substituted for the plasma or serum. It was found that the large molecular weight dextran sulphates did not affect the suspension-stability of any of these materials, in the absence of protein from the suspending medium. The addition of fibrinogen, plasma, or serum to mixtures containing the large molecular weight dextran sulphates produced agglutination of the particles with a corresponding order of efficiency. The small molecular weight dextran sulphate did not produce a similar effect.

Effect of the Dextran Sulphates on Erythrocytes

As shown in Experiment 1, none of the dextran sulphates affected the haematocrit value of blood to which it was added. It appeared, therefore, that these compounds, at the concentrations employed, exerted little or no osmotic effect on the red-cell. It was found that the presence of the I size dextran sulphate did not interfere with the ordinary haema-

tological investigations (red-cell count, haemoglobin estimation, etc.). The use of the I size dextran sulphate for keeping blood fluid for various laboratory procedures has been described by Hall and Ricketts (1952).

It is well known that the sedimentation rate of the erythrocytes is dependent upon rouleaux formation. It was first shown by Fåhraeus (1921) that alterations in the pattern of the plasma-proteins cause marked changes in the tendency to rouleaux formation and hence changes in the sedimentation rate. Increase of the macromolecular protein components, and especially an increased concentration of fibrinogen, the largest and most asymmetric of the normal plasma-proteins, results in acceleration of the sedimentation rate, under standardized conditions. It was shown by Ingelman and Halling (1949) that the addition to blood of macromolecular dextrans produced similar effects. This observation was confirmed and extended by Hardwicke, Ricketts, and Squire (1950), who also showed that other macromolecules were equally effective. They found that the phenomenon could be demonstrated most clearly after eliminating the effect of the normal sedimenting substance in plasma (fibrinogen) and after standardization of the red-cell volume at some given level (say 30% packed-cells). In such circumstances, increasing concentrations of any given macromolecule, with a molecular weight in excess of a certain critical level, produced corresponding increases in the sedimentation rate.

Since the large molecular weight dextran sulphates were known to precipitate the macromolecular proteins when added to plasma, it seemed likely that they would affect the sedimentation rate in a complex manner. Inhibition of sedimentation appeared likely to occur at concentrations sufficient to precipitate fibrinogen from whole blood. At higher concentrations, the "macromolecular effect" of the compounds themselves appeared likely to cause acceleration of sedimentation. It appeared probable that the small molecular weight dextran sulphates and heparin, which neither precipitated the plasma-proteins nor were large enough to produce direct effects, would fail to have any influence on the sedimentation rate. Experiments were designed to test these hypotheses.

Experiment 3.—In this experiment the various dextran sulphates and heparin were added in low concentrations to whole blood in which the concentration of fibrinogen and the sedimentation rate were known to be raised. The sedimentation rates of the samples containing the large molecular weight compounds were compared with that of a defibrinated sample of the same blood. The sedimentation rates of the samples containing heparin and the I size dextran sulphate were compared with an oxalated sample of the same blood.

Blood was taken from a patient suffering from an acute exacerbation of rheumatoid arthritis. The plasma contained 0.62 g.% of fibrinogen. 3 ml. aliquots of the blood were placed in each of a series of tubes containing 1 mg. of heparin or of the various dextran sulphates. A similar quantity of blood was added to a tube containing the balanced oxalate mixture of Heller and Paul. The blood was mixed well with the anticoagulants and transferred to Wintrobe tubes. Another sample of the same blood was defibrinated by constant stirring with a bent glass rod. The defibrinated blood was also placed in a Wintrobe tube. All the Wintrobe tubes were then suspended vertically in a water-bath at 25° C. and the fall of the column of red-cells was observed at five-minute intervals and plotted. The observed sedimentation rates (uncorrected) are shown in Fig. 3.

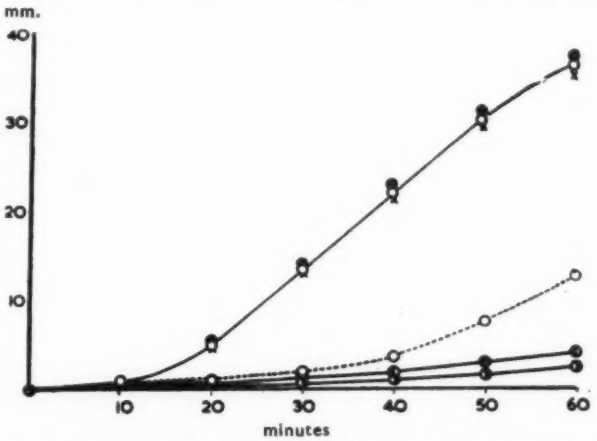

FIG. 3.—Effect on a pathologically raised erythrocyte sedimentation rate of the addition of the various anticoagulants as compared with the effect of defibrinating the same blood. X——X: Oxalate. ●——●: Heparin. ○——○: I size dextran sulphate. ○- - -○: E size dextran sulphate. ◐——◐: D size dextran sulphate. ◑——◑: Defibrinated blood.

It will be seen that there was close correspondence between the observed values for whole blood treated with oxalate, heparin, and the I size dextran sulphate. Practically no sedimentation occurred in the defibrinated blood or the blood treated with the D size dextran sulphate, while the curve for the E size compound (which showed less tendency to precipitate fibrinogen) occupied an intermediate position.

Experiment 4.—In this experiment the technique of Hardwicke *et al.* was used to investigate the sedimenting effect of high concentrations of the anticoagulants upon a standardized suspension of red-cells, in the absence of fibrinogen.

30 mg. each of heparin and of the three different dextran sulphates was weighed out in small glass bottles. A control bottle contained the normal amount (4.2 mg.) of the balanced oxalate mixture. 20 ml. of blood was taken from a normal human subject and defibrinated by constant stirring with a bent glass rod. The packed cell volume of the red-cells was adjusted to 30% by the addition of physiological saline and the diluted blood was distributed between the bottles. 3 ml. volumes of blood were added to the bottles containing heparin and the dextran sulphates, and 5 ml. was added to the oxalate bottle. Estimation of the sedimentation rate of the red-cells was carried out as in the previous experiment. The results obtained are shown in Fig. 4.

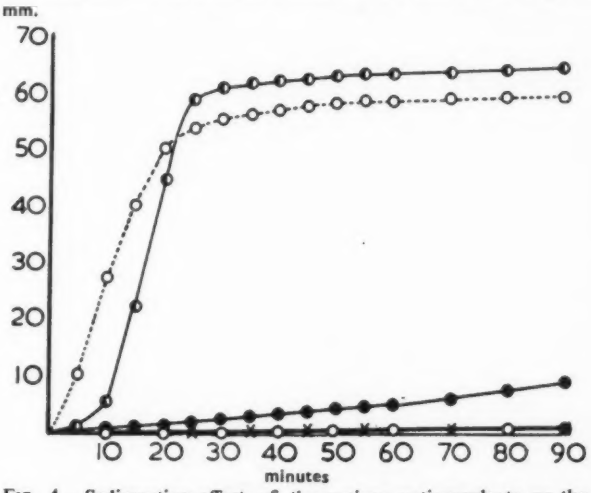

FIG. 4.—Sedimenting effect of the various anticoagulants on the erythrocytes in defibrinated blood. X——X: Oxalate. ●——●: Heparin. ○——○: I size dextran sulphate. ○---○: E size dextran sulphate. ◐——◐: D size dextran sulphate.

It will be observed that the sedimentation rate of the defibrinated blood containing 1% of the I size dextran sulphate did not differ significantly from that of the control containing the conventional concentration of balanced oxalate. The particular heparin sample used showed a slight sedimenting effect, but an enormously greater effect was obtained with the large molecular weight dextran sulphates. When the blood in the Wintrobe tubes was examined after sedimentation was complete, it was noted that rouleaux formation was markedly greater in amount in the tubes containing the D and E size dextran sulphates than in the control tube or in the tubes containing the I size dextran sulphate or heparin.

Effect of the Dextran Sulphates on Leucocytes

The effect of the dextran sulphates on the motility and phagocytic activity of leucocytes was investigated in the following manner:

Experiment 5.—Tenfold dilutions of heparin and of each of the dextran sulphates were made from stock solutions (containing 50 mg. per ml.) in 0.2 ml. volumes of mammalian Ringer solution. 0.8 ml. of blood, freshly drawn from a normal human subject, was added to each tube. In this way the final concentrations of heparin and of the dextran sulphates in the blood varied from 10 mg./ml. to 0.1 mg./ml. The tubes were incubated at 37° C. for fifteen minutes. At the end of this time thin smears and wet preparations were made from each tube. The wet preparations were examined immediately on a warmed stage under the phase-contrast microscope. Many leucocytes were observed to be entrained in the flocculent protein precipitates produced by the D and E size dextran sulphates, but the unagglutinated polymorphonuclear leucocytes and monocytes showed unimpaired motility at all concentrations of these compounds. Heparin and the I size dextran sulphate neither caused agglutination of the leucocytes nor affected their motility within the period investigated. When further samples were taken at intervals up to three hours, the leucocytes in the blood containing the dextran sulphates began to show the same degenerative changes that became noticeable in the blood kept fluid with heparin or oxalate. It was therefore evident that the dextran sulphates possessed no advantage over the latter as preservatives for the leucocytes.

Examination of the early stained films showed no morphological alterations of the leucocytes. The later films showed the presence in occasional monocytes of granules of basophilic material. This material stained metachromatically with toluidine blue. These cells were present in sufficient numbers to make it unlikely that they were degenerate basophilic leucocytes.

The experiment was repeated, but on this occasion two drops of a saline suspension of a coagulase-negative staphylococcus were added to each tube. The tubes were incubated at 37° C. for thirty minutes and once again smears and wet preparations were made. Examination of these showed that normal phagocytosis of the staphylococci had occurred at all concentrations of all the compounds.

Discussion of the in vitro *Effects of the Dextran Sulphates*

From the results of the experiments so far described, it is evident that the dextran sulphates, if of molecular dimensions greater than a certain critical limit, cause the aggregation of the formed elements of the blood. Earlier investigation of these large molecular weight compounds had shown that the particles of the insoluble complexes formed with fibrinogen tended to aggregate as floccules under suitable environmental conditions. Cohesion between the particles forming these floccules was assumed to be dependent upon weak intermolecular forces, since moderate thermal agitation of the molecules was sufficient to dissociate the floccules (Walton, 1952). Whether similar non-ionic forces would be adequate to bring about the association of the much larger formed elements of the blood is conjectural, but

there is some evidence that the agglutination of bacteria and of sensitized red-cells might be brought about by multiple non-ionic linkages resulting from antigen-antibody union at the cell-surfaces. It appears conceivable that the large molecular weight dextran sulphates might form complexes with the components of the plasma-protein film assumed to be present normally on the surface of platelets, erythrocytes, and leucocytes and thereby initiate conglutination of these structures in a similar manner.

Support for this hypothesis was provided by the observation that the large molecular weight dextran sulphates produced no agglutination of platelets, bacteria, or suspended particles of kaolin or graphite in the absence of protein in the suspending medium, but did bring about their aggregation on the addition of fibrinogen solution, plasma, or serum. Further support was provided by the observation that the small molecular weight dextran sulphate and heparin which did not form insoluble complexes with the plasma-proteins did not affect the stability of the formed elements of the blood.

The present findings are in keeping with those of Piper (1945a), who found that all the synthetic polysaccharide sulphates examined by him caused the agglutination of platelets. This was later associated with the fibrinogen-precipitating properties of the same compounds (Piper, 1945b). But in a subsequent communication Astrup and Piper (1946) implied that they were not entirely satisfied with this explanation, since they noted that an excess of cellulose trisulphuric acid or of chitin disulphuric acid still agglutinated platelets although visible precipitate formation was inhibited. On the other hand, in agreement with the present observations, they too noted that suspensions of kaolin or bacteria were only agglutinated in the presence of plasma.

It should be noted that although no precipitate is visible immediately with great excess of these compounds, yet it can be shown that interaction with fibrinogen still occurs, just as antigen-antibody union occurs in the " pro-zone " region of a precipitin titration, as shown by eventual visible precipitate formation even in this region (Walton, 1952). The suggested mechanism whereby particles become agglutinated by these compounds might still, therefore, be the same.

Complex formation with a plasma-protein fraction also occurs with heparin and with the I size dextran sulphate. Indeed the antithrombin activity of both compounds is dependent upon complex formation with a co-factor present in serum or plasma and suspected to be in the alpha-globulin or alpha-lipoprotein fraction. The complex formed with this fraction by either compound is, however, soluble under physiological conditions. Hence, it is suggested, its formation is not attended by any disturbance of the suspension stability of the plasma.

It has been reported by various authors that some samples of heparin do cause agglutination of platelets (Copley and Robb, 1941 ; Copley, 1948) and of leucocytes (Wilander, 1938). These observations were confirmed by Fidlar and Jaques (1948), who showed that carefully purified heparin did not behave in this manner. Quick, Shanberge, and Stefanini (1948) reported that the platelets in human blood were less sensitive to the agglutinating effect of heparin than the platelets in dog blood. Four preparations of British commercial heparin of varying degrees of purity (as judged by differences in anticoagulant activity) were examined personally, but failed to produce agglutination of the platelets in human blood. The heparins used were all stated to originate from beef lung. The investigation was not extended to include heparin derived from other tissue, nor was the effect on blood or blood cells from other species investigated.

It has been shown that purified heparin, at anticoagulant concentrations (1 mg. per 5–50 ml. blood), has no appreciable effect on the sedimentation rate (Wilander, 1938 ; Nielsen, 1942), but conflicting results were obtained with earlier crude preparations (Enocksson, Gjertz, Schnell, and Torgersruud, 1936) and with high concentrations (1 mg. or more per ml. blood) of commercial heparin (von Kaulla, 1939 ; Sappington and Gillis, 1941).

Even purified heparin is acknowledged to be polydisperse with regard to molecular size (O'Keef, Russo-Alesi, Dolliver, and Stiller, 1949). The molecular weight of 17,000 obtained by Grönwall *et al.* (1945) and by Jensen, Snellman, and Sylvén (1948) was calculated to be the average of the molecules present in the preparations. It has been shown by Jorpes and Gardell (1948) that a common contaminant of commercial heparins is chondroitin sulphate, a compound of considerably larger molecular weight than heparin. It is tempting to suggest that the presence, in amounts varying from one sample to the next, of contaminants or impurities of this sort might have brought about, by a mechanism similar to that put forward for the large molecular weight dextran sulphates, the conflicting and anomalous results referred to above.

It will be shown in a subsequent communication that the effects produced by the large molecular

weight dextran sulphates on the blood cells *in vitro* also occur *in vivo* in experimental animals and are largely responsible for the greater toxicity of the D and E size compounds as compared with the I size compound or heparin.

Summary

1. A comparison was made between heparin and a series of sulphuric esters prepared from dextrans of varying molecular weight, in respect of the effects of these anticoagulants on the formed elements of the blood.

2. The dextran sulphates were found to be divisible into two broad groups: (*a*) Compounds with molecular weights in excess of 40,000 which differed from heparin in causing platelet and white-cell agglutination and variable effects on the erythrocyte sedimentation rate. (*b*) Compounds with an average molecular weight of about 7,500 which resembled heparin in producing no appreciable effects on the blood cells.

3. The difference between the two groups of dextran sulphates were considered to be dependent ultimately upon differences in the nature and behaviour of the non-ionic complexes which they formed with certain of the plasma-proteins. The large molecular weight group formed complexes mainly with fibrinogen. The small molecular weight compounds, like heparin, formed complexes with an incompletely identified fraction of the plasma-proteins, suspected to be the alpha-globulin.

4. The complexes formed with fibrinogen by the large molecular weight dextran sulphates were insoluble under physiological conditions. The precipitation of fibrinogen in this manner was shown to interfere with the suspension stability of the formed elements in the blood. As a result, the platelets and white-cells were found to agglutinate and the red-cells to aggregate in rouleaux and clumps. The possible detailed mechanisms of these changes are discussed.

5. On the other hand, the complexes formed by the small molecular weight dextran sulphates were soluble in plasma under physiological conditions. No agglutination of the platelets or white-cells occurred and there was no appreciable effect on the erythrocyte sedimentation rate.

It is a pleasure once more to acknowledge the advice and assistance of my colleague, Dr. C. R. Ricketts, in various aspects of this investigation. I am grateful to Professor J. R. Squire for his encouragement, for his helpful criticisms, and for his assistance with the manuscript.

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NICOTINE-LIKE ACTIONS IN AURICLES AND BLOOD VESSELS AFTER DENERVATION

BY

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The work to be described in this paper is a continuation of observations made on the action of nicotine and acetylcholine in the isolated auricles of the rabbit heart and in the perfused vessels of the rabbit ear (Kottegoda, 1953a, 1953b). Nicotine and acetylcholine were found to stimulate the auricles in the presence of atropine, and the stimulant action was blocked by hexamethonium. Similarly nicotine was found to constrict the vessels of the rabbit ear; acetylcholine in the presence of atropine or when the perfusion had continued for 24 hours had the same effect. This constrictor action was blocked by hexamethonium; in the presence of benzylimidazoline (Priscol) the constrictor action was reversed to a dilator action, and this dilator action was also blocked by hexamethonium. The observations suggested that the actions of nicotine and of acetylcholine were indirect and were due to the liberation of an adrenaline-like substance. Because of these and various other findings discussed later further experiments were undertaken to test the action of nicotine and acetylcholine on auricles and on vessels which had been denervated some time previously.

For experiments on auricles, cats were used in place of rabbits, as the former were found to stand the operation better. A few auricles from normal cats were tested with nicotine and acetylcholine in the presence of atropine; it was found that they behaved like rabbit auricles towards these substances.

METHODS

Cat Auricles. Both stellate ganglia were removed from cats under ether and pentobarbitone sodium (30 mg./kg. body weight intraperitoneally). After 12 to 14 days the animals were killed, the auricles dissected out, and the response to acetylcholine and nicotine tested according to the method described earlier (Kottegoda, 1953a).

Rabbit Ears. The sympathetic supply to the rabbit's ear consists of post-ganglionic fibres mainly from the superior cervical ganglion, but also of fibres from the stellate ganglion which do not pass through the superior cervical ganglion (Feldberg, 1926). Both ganglia on one side were removed under ether and pentobarbitone sodium anaesthesia. A very great portion of the sensory supply of the rabbit ear is provided by the great auricular (C2 and C3) and great occipital (C2 and partly C3) nerves. For sensory denervation of the ears, one-third to half-an-inch of each of these nerves was removed proximal to the insertion of the ears. The perfused vessels of the isolated ears were tested with nicotine and acetylcholine according to the method described by Burn and Robinson (1951), in which Stephenson's recorder (1948) was used.

RESULTS

Isolated Cat Auricles.—Three auricles were tested with nicotine and acetylcholine in the presence of atropine 12–14 days after denervation. Nicotine was found to increase both the rate and amplitude of the auricular beat (Fig. 1). This stimulant action,

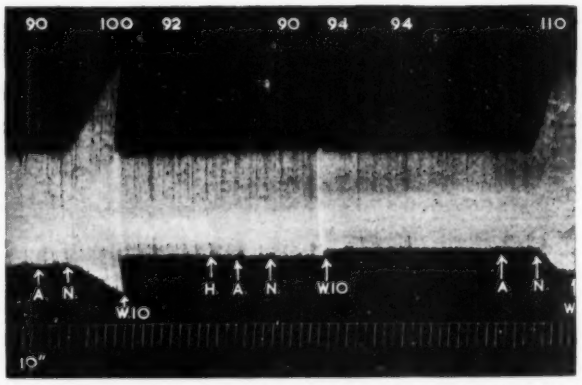

FIG. 1.—Spontaneous contractions of cat auricles which had been sympathetically denervated 2 weeks earlier, showing the stimulant action of nicotine after atropine. Bath volume 40 ml. At A 10 μg. atropine sulphate was added after which 0.4 mg. nicotine hydrogen tartrate was added at N. At W the bath was changed 10 times, and at H 2.5 mg. hexamethonium bromide was added followed by 10 μg. atropine sulphate. Nicotine (N) was then without effect. After washing out the bath at W the stimulant action of nicotine after atropine returned. Figures at top of tracing denote number of auricular beats per minute.

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like that observed in normal auricles, was suppressed by hexamethonium and returned after the hexamethonium was washed out. Similar effects were observed with acetylcholine.

Rabbit Ears after Sympathetic Denervation.—Three isolated ears were examined 1 to 3 weeks after removal of the sympathetic ganglia. In these nicotine caused a marked constriction only, or one which was followed by a small dilatation. Hexamethonium always blocked this constrictor action (Fig. 2), which reappeared when the hexamethonium was washed out. In fresh preparations acetylcholine caused only a dilatation; but if atropine was first injected or if larger doses of acetylcholine were given after the preparation was a few hours old, a constriction was produced which was blocked by hexamethonium. The constrictor action of acetylcholine returned gradually as the hexamethonium was removed. In previous experiments on isolated rabbit ears (Kottegoda, 1953b) the constriction caused by a given dose of nicotine or acetylcholine increased as the perfusion was continued. In the ears in which the sympathetic fibres had degenerated, the initial constrictor response to these substances was always greater than in normal ears, and there was no tendency for the constriction caused by a given dose to increase with time. The responses remained more or less the same or declined as the perfusion was continued Fig. 3.

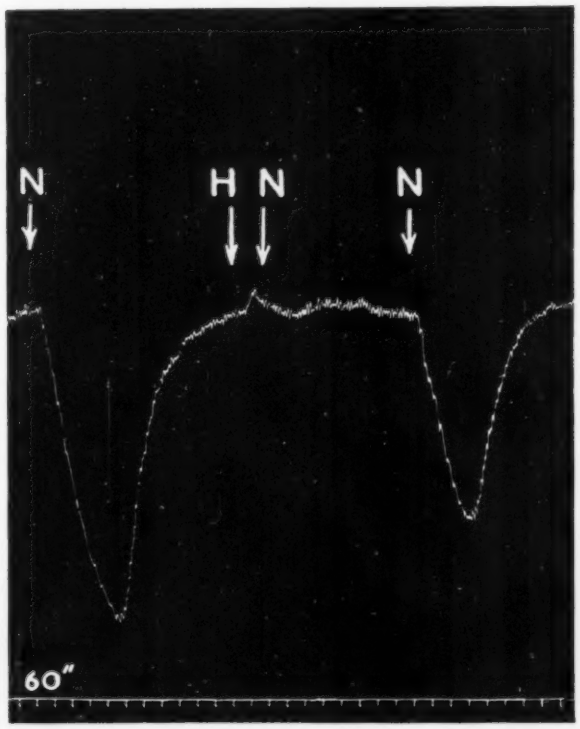

FIG. 2.—Outflow record from perfused rabbit ear, sympathetically denervated 3 weeks earlier. At N injection of 10 μg. nicotine hydrogen tartrate caused a reduction in outflow. At H 100 μg. hexamethonium bromide was injected, followed by the same dose of nicotine at N. In the presence of hexamethonium, nicotine had no effect. After some time, as the hexamethonium was washed away nicotine (N) again caused a reduction in outflow. (A fall in the record in this and subsequent figures indicates a reduction in outflow.)

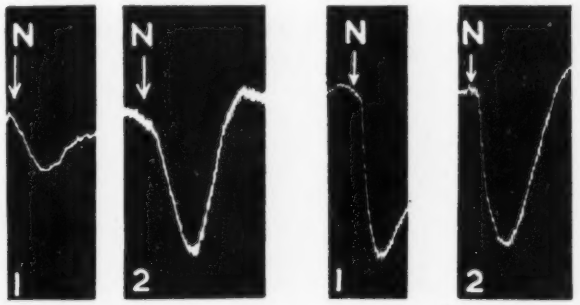

FIG. 3.—Outflow from perfused rabbit ear. (*a*) Normal ear. Effect of 20 μg. nicotine hydrogen tartrate at N on the first day (1) and the same dose of nicotine on the second day (2). Note the bigger reduction in outflow on the second day to the same dose. (*b*) Sympathetically denervated ear. Effect of 10 μg. nicotine hydrogen tartrate (1) on the first day and (2) of the same dose on the second day. The response does not now increase as the perfusion is continued.

Rabbit Ears after Sensory Denervation.—Four ears were examined 1 to 4 weeks after sensory denervation. In these, too, nicotine caused a constriction which was often great. This constriction was sometimes followed by dilatation. Again, hexamethonium suppressed the constriction caused by nicotine. Acetylcholine injected early in the perfusion caused a dilatation only, but after atropine it caused a constriction which, like that caused by large doses of acetylcholine in the older preparations, was blocked by hexamethonium (see Fig. 4). Once the latter was washed away the constrictor action of acetylcholine returned.

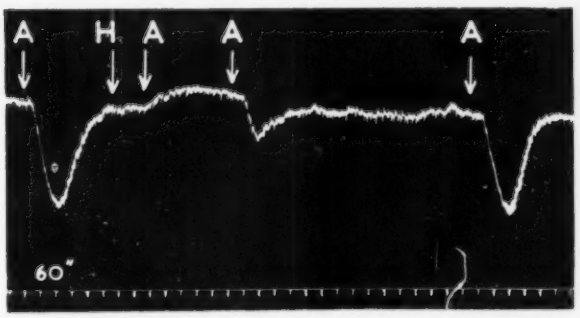

FIG. 4.—Outflow from rabbit ear 10 days after sensory denervation. 2nd day perfusion. At A 40 μg. acetylcholine bromide was injected. At H 100 μg. hexamethonium bromide was injected, followed by the same dose of acetylcholine at A. Now the acetylcholine was without effect. Note the return of the effect of acetylcholine as the hexamethonium is washed out.

Discussion

The well-recognized peripheral effects of nicotine occur at the motor end plate and in ganglia. Several phenomena have now been described by various workers which, in one respect or another, are anomalous. The first of these is the stimulant action of nicotine (Heymans, Bouckaert, and Dautrebande, 1931) and of acetylcholine (v. Euler, 1938) on the carotid body. Moe, Capo, and Peralta (1948) found that tetraethylammonium blocked the action of these substances, but did not modify the response to hypoxia and the effect of cyanide. Ginzel, Klupp, and Werner (1952) and Douglas (1952) showed that hexamethonium behaved like TEA. The second is the pilomotor response which occurs in the skin of man and of the cat after the intracutaneous injection of nicotine or of acetylcholine. This was described in 1940 by Coon and Rothman. The third is the stimulant action of acetylcholine in the heart of the cat or rabbit after treatment with atropine, accompanied by the release of an adrenaline-like substance. This was described by Hoffmann, Hoffmann, Middleton, and Talesnik in 1945. A similar action of acetylcholine and of nicotine has been observed in the isolated rabbit auricles and found to be blocked by hexamethonium (Kottegoda, 1953a). The fourth is the stimulant action of acetylcholine and of nicotine on sensory nerve endings in the skin described by Brown and Gray (1948). Douglas and Gray (1953) have recently shown that this action, like that on the carotid body, is blocked by hexamethonium, but that the receptors in the skin still discharge impulses in response to touch. The fifth is the inhibitory action of nicotine on the isolated intestine in the presence of atropine, which was described by Ambache and Edwards in 1951. The last is the constrictor action of nicotine and acetylcholine in the rabbit ear vessels recently shown by one of us to be abolished by hexamethonium (Kottegoda, 1953b).

The actions of nicotine on the carotid body receptors and on the sensory receptors of the skin are presumably not ganglionic actions, and since they are blocked by hexamethonium, it follows that the action of hexamethonium can exclude other actions of nicotine than those exerted on ganglia. Ambache and Edwards (1951) believed that the inhibitory action of nicotine on the intestine was exerted on ganglion cells of the myenteric plexus which possessed adrenergic neurones. Evans and Schild (1953) found, however, that a transient inhibitory action of nicotine was obtained in ganglion-free specimens of cat jejunum, particularly in preparations stimulated by eserine and acetylcholine.

In the heart, on the other hand, the stimulant action of nicotine seems more likely to be an action on ganglia, or at least an action on tissue able to liberate an adrenaline-like substance, some vagal preganglionic fibres functioning perhaps like the splanchnic terminations in the adrenal medulla. Middleton, Middleton, and Toha (1949) have shown that vagus stimulation liberates an adrenaline-like substance from the isolated heart after treatment with atropine, so that the acceleration occurs after a physiological stimulus as well as when nicotine or large doses of acetylcholine are applied. Our experiments show that the stimulant action of nicotine and acetylcholine is present after degeneration of the sympathetic fibres, and is therefore probably related to this vagus acceleration.

There remains for consideration the pilomotor phenomenon of Coon and Rothman, and the constrictor action in the vessels of the rabbit ear, These two effects must have a different mechanism. since the pilomotor action disappeared after degeneration of the sympathetic fibres. Coon and Rothman came to the conclusion that nicotine stimulated some of the terminations of the post-ganglionic sympathetic fibres and then by an axon reflex caused erection of the hairs. The arguments for this view were that the intracutaneous injection of nicotine or acetylcholine caused the pilomotor response after section of the mixed nerve supply, but not after degeneration of the sympathetic fibres, and not in the presence of concentrations of procaine too low to have an analgesic action, and therefore too low to paralyse the sensory fibres. Their conclusion is of such importance that it would seem desirable that the experiments after sympathectomy should be repeated.

In the rabbit ear vessels we have found that the constrictor action of acetylcholine seen after giving atropine or after prolonged perfusion, and the constrictor action of nicotine, remain unaffected by degeneration of all sympathetic fibres and also by degeneration of the sensory fibres. Hence the constrictor effect cannot be explained by an axon reflex involving either sympathetic or sensory channels. It is an effect which seems to be due to the release of an adrenaline-like substance since in the presence of benzylimidazoline (Priscol) both acetylcholine and nicotine cause dilatation. Moreover, the action, whether constrictor, or after reversal by benzylimidazoline dilator, is abolished by hexamethonium. Evans and Schild say that block by hexamethonium may be due to the action of hexamethonium on the end organ, and that therefore caution is required in drawing a conclusion. However, hexamethonium in very high doses

does not modify the constrictor action of adrenaline, and we have found that when the postganglionic fibres to the rabbit ear vessels are stimulated in the preparation described by Gaddum and Kwiatkowski (1938), the constriction obtained remains unchanged after the injection of hexamethonium. (This result indicates in addition that there are no ganglia on the course of fibres arising from the superior cervical ganglion.)

Hence abolition by hexamethonium suggests that acetylcholine and nicotine cause constriction either by stimulating ganglia with adrenergic neurones or by stimulating tissue resembling chromaffin tissue. At this point histological evidence is required which is at present not available. In the intestine a terminal reticulum has been described by Stöhr (1932, 1934) and by Reiser (1933) and a sympathetic ground plexus by Boeke (1940). These do not degenerate after removal of the ganglion cells. Something of the same kind may be present around the vessels. Nelemans (1948) has described structures resembling ganglia in the nerve plexuses surrounding the smallest blood vessels of the frog's tongue. They persisted after degeneration of the sympathetic fibres and of the somatic fibres. Our observations suggest that the rabbit's ear offers a tissue for study where histological and functional changes may be correlated.

Summary

1. The stimulant action of nicotine and acetylcholine on the atropinized cat auricle has also been found in preparations in which the sympathetic fibres have degenerated. Hexamethonium abolishes this effect in the normal as well as in the denervated auricle.

2. In isolated perfused rabbit ears deprived of their sympathetic and sensory supply 1–4 weeks previously, nicotine and acetylcholine (the latter after atropine or on the second day of perfusion) exert a vasoconstrictor effect as they do in normal ears. Hexamethonium blocks this action in both preparations.

3. The significance of these results in relation to the possible occurrence of peripheral ganglia with adrenergic neurones or tissue resembling chromaffin tissue is discussed.

We wish to express our thanks to Professor J. H. Burn for his advice and guidance throughout this work.

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THE AFFERENT PATHWAY OF THE BEZOLD REFLEX: THE RIGHT VAGAL BRANCHES IN CATS

BY

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In 1867 von Bezold and Hirt showed that intravenous injection of veratrum alkaloids caused a fall of blood pressure and heart rate, which was abolished by cutting the vagi. They attributed the phenomenon to an action on receptors in the heart.

The principal physiological interest of the Bezold reflex lies in the nature of these receptors, and before these can be identified the afferent pathway of the reflex must be established with certainty. In 1939 Jarisch and Richter investigated the afferent pathway of the reflex in cats. On the right side they concluded that it lay among a group of nerves which leave the vagus just as it crosses the right subclavian artery, and which run beneath the aortic arch toward the ventricles; on the left side it was not clearly defined. However, Amann and Schaeffer (1943) observed an increase in the discharge frequency of vagal branches *from the right atrium* after administration of veratrine, and in 1948 Jarisch and Zottermann concluded from similar observations that these branches contain the Bezold afferent nerve fibres. There is therefore a discrepancy in the evidence for the afferent pathway of the reflex.

This paper describes experiments to identify the afferent nervous pathway of the Bezold reflex on the right side in cats. The effects of stimulation of the central ends of these nerves, and of the application of a cold block to the vagus in the neck are also described.

METHODS

Cats were anaesthetized with chloralose, 60 mg./kg. The chest was opened along the midline and artificial ventilation was applied. The blood pressure was recorded with a mercury manometer from the carotid artery. Intravenous injections were made from a cannula in the right external jugular vein.

The vagi were cooled by applying hollow thermodes through which alcohol was circulated from a reservoir in an ice-salt mixture. The thermodes, which were covered with silver foil, were in contact with the nerve for a distance of 1 cm., and their temperatures were recorded from thermocouples in contact with their surface. They were similar to those used by Dawes, Mott, and Widdicombe (1951), and the results obtained are therefore comparable.

Branches of the right vagus running to the right atrium were cut as near to the heart as possible, and their central ends were stimulated with rectangular current pulses using a constant current output. The compound action potentials of the right cervical vagus were recorded with a conventional resistance-capacity coupled differential amplifier having a time constant of about 1 second. They were displayed on a cathode-ray oscilloscope together with time marks of 0.1 and 1 msec. from a crystal-controlled oscillator.

The veratridine used was the same as that used by Dawes (1947) and Dawes, Mott, and Widdicombe (1951), and was obtained from Professor O. Krayer.

RESULTS

The Afferent Pathway of the Bezold Reflex on the Right Side.—Jarisch and Richter (1939) used veratrine, a mixture of alkaloids which is rather liable to cause tachyphylaxis, in their experiments to locate the afferent pathway of the Bezold reflex. In the experiments to be described one of the pure veratrum alkaloids, veratridine, was used throughout. Previous work (Krayer and Acheson, 1946) has shown that this alkaloid does not cause tachyphylaxis so readily, and that its duration of action is short. Injections of 10–25 μg. repeated every 10 min. have given satisfactory responses.

Section of the upper right vagal branches to the heart (Fig. 1, n. I) did not abolish the Bezold reflex, even though the left vagus had been cut. Subsequent injection of the same amount of veratridine into the ascending aorta did not cause a fall of blood pressure and heart rate; hence the effect observed after section of the left vagus and n. I on the right side was not due to a central action. The afferent pathway must therefore lie principally in n. II or n. III (Fig. 1).

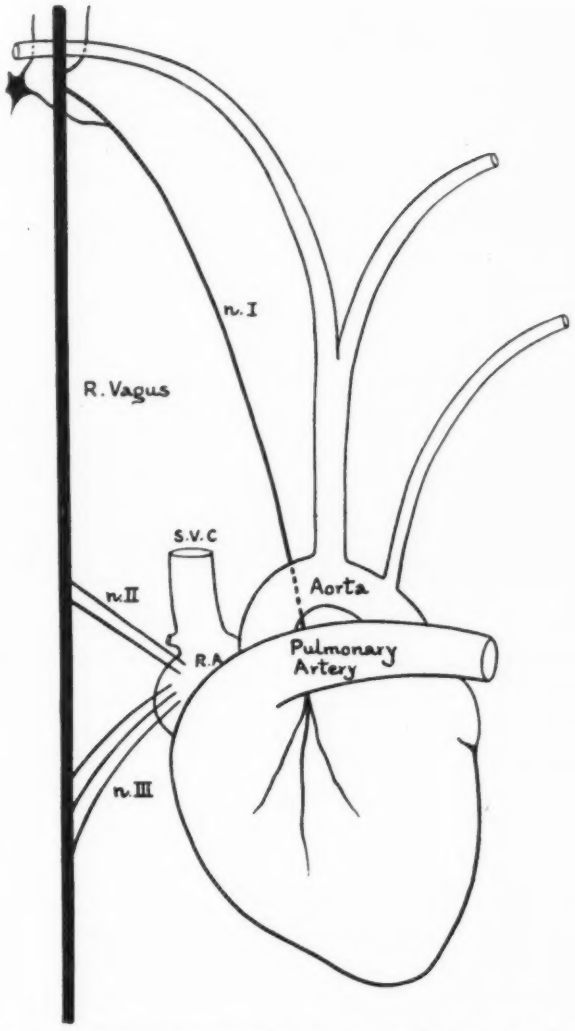

FIG. 1.—Diagram of the distribution of the cardiac branches of the right vagus in the cat.

Although it seems fairly certain that a proportion of the Bezold afferent nerve fibres must run in n. II, which also carries efferent cardio-inhibitory fibres to the right atrium, it is clear that n. III is also of importance. A typical experiment designed to test this point is illustrated in Fig. 2. The first injection of veratridine (A) was given while the *right* vagus was cooled to 8° C., a temperature at which the afferent fibres, but not all the efferent fibres, are blocked (Dawes, Mott, and Widdicombe, 1951). There was only a small fall of blood pressure and heart rate, suggesting that in this particular cat most of the afferent nerve fibres ran in the right vagus. Twenty-five sec. after the injection the right vagus was warmed, and there was an immediate further large fall of blood pressure and heart rate. The right vagal branches, n. I and II, were then cut, and the trachea was scraped to eliminate other branches. The next dose of veratridine (B) was injected while the *left* vagus was cooled to 8° C. There was a considerable fall of blood pressure and heart rate, showing that some afferent nerve fibres were still present on the right side. The left vagus was warmed about 50 sec. after the injection, and there was a further fall of blood pressure. The major part of n. III was then cut, including those nerves running across the right bronchus and through the lymph node at the origin of the latter from the trachea. With the left vagus at 8° C., injection of veratridine still caused a fall of blood pressure and heart rate (C). Finally, a number of nerve fibres running across the right pulmonary vein were destroyed. With the left vagus at 8° C., veratridine now caused a small rise of blood pressure (D). When the left vagus was warmed there was an immediate fall of blood pressure and heart rate, from which it can be seen that the cat had not become appreciably tachyphylactic during the experiment.

Twelve experiments of this type were performed; in six of these the afferent nerve fibres on the right side were destroyed before the cat became relatively insensitive to injections of veratridine. The fall of blood pressure was never completely abolished until n. III had been destroyed; often there was little reduction in the effect when n. I and II were cut. In all but one cat injection of veratridine caused considerable reflex bradycardia while the *right* vagus was cooled to 8° C., indicating that a number of afferent nerve fibres also run in the left vagus.

Electrical Stimulation of the Right Vagal Cardiac Nerves.—Jarisch and Zottermann (1948) caused a fall of blood pressure and heart rate by stimulating the central ends of n. II, which Jarisch and Richter (1939) had thought carried mainly efferent cardio-inhibitory nerve fibres. These observations have been repeated, and the temperature at which the afferent impulses are blocked has been determined by cooling the right vagus in the neck.

The largest vagal branch to the right atrium (n. I) was cut as near to the heart as possible and freed from surrounding tissue for 2 cm., to prevent direct current spread to the heart. In 18 cats stimulation of this nerve caused a fall of blood pressure and heart rate; occasionally this was preceded by a slight rise in blood pressure. In a few cats in earlier experiments only a rise of blood pressure with no consistent change of heart

rate was observed ; the possibility that the nerve had been damaged cannot be excluded.

Stimuli were delivered at a frequency of 20–100 per sec. (usually 77 per sec.) for 5 sec. In most cats the minimum current required to elicit a fall of blood pressure was 0.2 mA with a duration of 5–7 msec. With currents of 0.3–0.4 mA the duration could be reduced to 1.0–0.5 msec. The application of still larger currents did not reduce the duration necessary to elicit a fall of blood pressure.

In eight successful experiments the right vagus was cooled in the neck while the nerve (n. II) was stimulated. In five cats the fall of blood pressure and heart rate was abolished by cooling the right vagus to 10–12° C., and in the other three cats at 8–10° C. The cooling characteristics of these nerve fibres are therefore the same as those of the Bezold afferent nerve fibres. In three cats the *left* vagus was cooled subsequently in order to discover at what temperature the efferent cardio-inhibitory fibres were blocked, when stimulated reflexly. The bradycardia disappeared when the vagus was cooled to between 6 and 8° C.

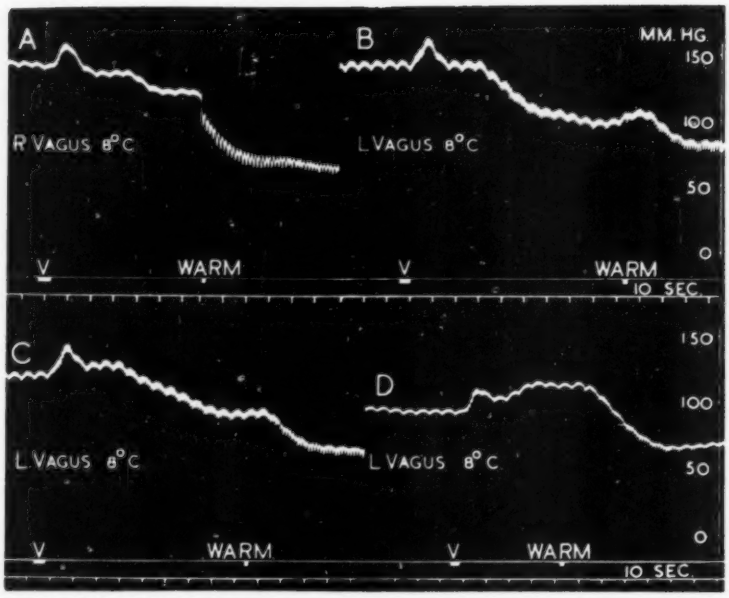

FIG. 2.—Cat, 2.4 kg.: chloralose anaesthesia. Chest opened; artificial respiration; record of carotid arterial blood pressure. At V 25 μg. veratridine was injected intravenously, at intervals of approximately 10 minutes. The right or left vagus was cooled to 8° C. for 2 minutes before each injection; the vagus was warmed at the point indicated. Between A and B, n. I and II (Fig. 1) were cut; between B and C part of n. III, and between C and D the remainder of n. III were cut.

In 26 cats compound action potentials were recorded from the intact vagus in the neck, while the central ends of vagal branches from the right atrium (n. II) were stimulated. The blood pressure was also recorded in order to gauge the effect of stimulation. The experiments were performed with the vagus exposed to air, and so the conduction velocities observed are probably too low. With this reservation, the conduction velocity of the peak elevation was 22–33 m./sec. in five cats. Occasionally a second, smaller elevation of the compound action potential followed the first. It was more prolonged, and the stimulus strength required to cause its appearance was usually well above that required to cause a fall of blood pressure.

DISCUSSION

There are several difficulties in designing a satisfactory type of experiment to define the afferent pathway (or pathways) of the Bezold reflex on the right side in the cat :

1. Successive injections of the veratrum alkaloids cause diminishing effects (tachyphylaxis).

2. Veratrum alkaloids cause a fall of blood pressure and heart rate not only by an action on receptors in the heart (the Bezold reflex), but also by a central action whose precise mechanism (there may be more than one) has not been systematically analysed. While it is certainly possible to limit the site of action of veratridine by injection into the coronary arteries, it is not a practical proposition to combine the extensive dissection which this involves with that required for progressive nerve section. Therefore, doses of veratridine must be chosen which are large enough to excite only the receptors for the Bezold reflex but not to act centrally. This choice may be checked either by injection of veratridine into the ascending aorta (when it should have no effect on blood pressure or heart rate) at the beginning of the experiment, or by intravenous injection when the vagi are cut at the end of the experiment. Both tests have been employed in the work described above.

3. Progressive section of branches of the right vagus not only interrupts afferent nerve fibres, but also efferent cardio-inhibitory nerves. Hence a reduction in the fall of blood pressure and heart rate may be due to section of either afferent or efferent nerves. This makes the attribution of the afferent pathway to a particular vagal branch or branches a difficult problem, so far as nerve-section experiments are concerned.

4. It is equally difficult to interpret action-potential records from vagal branches from the heart, since the veratrum alkaloids are now known to excite many types of nerve ending.

The results obtained in this paper differ in a number of details from those recorded by Jarisch and Richter (1939), but it is believed that the discrepancies can be explained by the fact that the latter were using large doses of veratrine (sometimes as much as 200 μg.), and were not at that time fully aware that such large doses might have a central action as well as an action on receptors in the heart. It was not until 1943 that Krayer, Wood, and Montes showed that veratridine injected into the isolated head circulation caused bradycardia. There is a further point. It is now well known that intravenous injection of *large* doses of the veratrum alkaloids cause a rise of blood pressure when the vagi are cut, partly at least because of a liberation of adrenaline from the adrenal glands (Mendez and Montes, 1943). Some of Jarisch and Richter's (1939) tracings show evidence of this rise of blood pressure, which implies that the doses used must have been sufficient to cause a very widespread and complicated action upon the cardiovascular system, to a degree that would render the analysis of the effects of progressive nerve section most difficult. In these circumstances it is not surprising that different conclusions have been reached.

In spite of the precautions which were observed in the experiments described in this paper it will be seen that the afferent pathway on the right side of the cat has not been localized to a single vagal branch. So far as these experiments allow, we must conclude that the afferent pathway is widespread, scattered over a number of fine branches which run towards the heart between and around the great veins and the right bronchus. Some of these branches also carry the efferent cardio-inhibitory branches to the heart. There is as yet no effective method of distinguishing between the Bezold afferent nerve fibres and the cardio-inhibitory efferent nerve fibres so far as the reaction of the cat's blood pressure and heart rate to veratridine is concerned.

Summary

The branches of the vagus, which carry the afferent nerve fibres for the Bezold reflex, are widely distributed on the right side in cats.

Stimulation of their central ends causes a fall of blood pressure and heart rate which is blocked by cooling to 8–10° C.

It is a pleasure to thank Dr. G. S. Dawes, who suggested these experiments, for his help throughout the work, which was carried out during the tenure of a grant from the Medical Research Council.

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A COMPARATIVE STUDY ON THE METABOLISM OF *ISO*NICOTINIC ACID HYDRAZIDE AND OF *ISO*NICOTINOYL-HYDRAZINOMETHANESULPHONIC ACID IN THE RAT

BY

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The discovery of the antituberculous activity of *iso*nicotinic acid hydrazide (isoniazid) has stimulated work intended to find new derivatives equally or more active, but less toxic than isoniazid itself. As reported in a previous paper (Ceriotti and Franceschini, 1953), we found that N'-*iso*nicotinoylhydrazinomethanesulphonic acid* (INHM) had these advantages.

During the study of the activity of this drug, a discrepancy was observed between the *in vitro* and the *in vivo* activity (Ceriotti and Franceschini, 1953; Ceriotti, 1953a). The *in vitro* activity of INHM was somewhat lower, the *in vivo* higher, when compared with a corresponding amount of isoniazid.

This interesting feature could be ascribed either to a difference in the mode of action of the two drugs or to a difference of their behaviour when introduced into the organism. Although some difference in the mode of action of the two drugs could not be excluded (Ceriotti, 1953b), the more likely explanation seemed to depend upon some quantitative or qualitative difference in their metabolism.

To compare the fate of isoniazid and INHM in the organism, and to study their physico-chemical properties, the present researches were accordingly planned, in the hope that they might throw light on the immediate problem and have some significance also in the field of general pharmacology.

MATERIAL AND METHODS

The isoniazid and the INHM used throughout our experiments were prepared by the chemical department of our Institute (Table I). For most of the experiments, the Ca salt of INHM was used. When small volumes at high concentration had to be used, for intratracheal and intracranial administration for instance, the Na salt was employed, owing to its higher solubility in water. The amounts of INHM were calculated on the basis of the isoniazid content of this compound.

The concentrations of the drugs were always determined both by chemical and by microbiological methods. It was thus possible to evaluate the degree and the rate of inactivation of the two compounds.

The preparation of samples for assay was different according to the material used and the method applied. No special preparation was required for urine specimens, which were simply diluted on the basis of their presumed content. A series of assay dilutions were made previously and then, from the results of these first determinations, the samples were brought to a concentration resembling as closely as possible that of the standard solution, that is, 2.5 μg./ml., corresponding to the middle part of the average standard curve.

For blood, the determinations were made on serum heated for 10 minutes in a boiling water-bath in centrifuge tubes; the tubes were cooled under running water, some quartz sand added, and they were then centrifuged at 4,000 rev./min. for half an hour. The addition of quartz sand assisted expression of the liquid from the abundant protein precipitate. The clear supernatant fluid was used for the determinations.

Intestine and lungs were carefully freed of fat and connective tissue, minced, mixed with quartz sand, frozen with solid carbon dioxide and triturated to a fine powder. They were then extracted with distilled water by boiling for 10 minutes. The amount of water corresponded to about ten times the weight of the sample used; it could be more or less according to the amount of drug present, giving a lower or higher dilution. An alternative method was also used, which gave similar results. The powdered organs were extracted by repeated washings with distilled water at room temperature, the washes combined and heated for 10 minutes in a boiling water-bath, and then centrifuged again. For intestine, the supernatant fluid was sufficiently free from proteins to allow direct use for the determination.

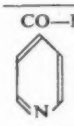
For lungs, boiling for 10 minutes was not enough to precipitate the proteins consistently. As the presence of proteins influences the results of the determination by the microbiological test considerably, additional treatment was required. To four parts of

*See Table I for structural formula. This compound has also been called *iso*nicotinic acid hydrazide methanesulphonate.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF ISONIAZID AND INHM

I.T. is the iodometric titre, which is the amount in grammes corresponding to 1 ml. of a 0.1 NI solution

	M.W.	m.p.	$E_1^{1\%}$cm. (H_2O)	Log. ε	I.T.		Solubility (20° C.)		
					Theor.	Found	Water	Ethanol	Acetone
 Isoniazid	137·1	169–171°	309 (268·5 mμ)	3·628	0·00343	0·00348	11·5%	2·4%	2%
INHM Na salt	271·2		158 (263 mμ)	3·631	0·00339	0·00346	12%	0·08%	0·0008%
INHM Ca salt	536·5	205–210°	159 (263 mμ)	3·630	0·00336	0·00346	2%	0·024%	0·0012%

extract, prepared as described above, by hot or cold procedure, one part of acetate buffer solution 0.01M at *p*H 4.4 was added, the tubes shaken well and kept for 5 minutes at 60° C. The protein precipitate was centrifuged out. To the limpid supernatant fluid, three parts of phosphate buffer 1/15M at *p*H 7 were immediately added. By this treatment the original extract was diluted twofold.

Several recovery experiments were made to test the validity of these methods of determination and extraction for urine, blood, intestine, and lungs of rats. For serum, intestine, and lungs the recovery is complete by chemical methods, and lies between 80 and 90% with the biological determination. The recovery from urine is complete for isoniazid when urine is diluted tenfold, whereas for INHM only 72% is recovered even at a dilution of 1:200. This is possibly due to the presence of some substances which counteract the action of INHM more than that of isoniazid. It is, of course, a disadvantage in so far as the exactness of the method is concerned; it poses however a very interesting biological problem, which will be discussed later. No special treatment was necessary for chemical determination in urine. For blood and organ extracts the preparation was, for the first part, the same as for the biological determination. However, as complete deproteinization is of the utmost importance, blood and intestine extracts were also always treated with acetate buffer. Both the microbiological and chemical determinations were always performed on the same sample.

Microbiological Determination.—For the microbiological determination, a plate method was worked out, using the strain of *M. tuberculosis* ATCC 607. Sensitivity with this plate method reached 0.5 μg. isoniazid per ml. The method has already been described (Ceriotti, 1952a and b). It has been improved by using an ATCC 607 strain made resistant to 2,000 μg./ml. of streptomycin. This allows the determination of INH and its derivatives in organic liquid extracts in the presence of large amounts of streptomycin.

The micro-organism for inoculation is cultivated in Youmans medium with the addition of 1% Tween 80, to allow diffuse growth, at 37° C. for 24 hr. with shaking. The culture is then diluted to an optical density of 0.050, at 650 mμ, in test-tubes of 11 mm. in diameter, using the Coleman spectrophotometer. Two ml. of the bacterial suspension is thoroughly mixed with 80 ml. of the agar medium, kept liquid at 45° C. and poured on to a supporting layer of 240 ml. of the same medium on a rectangular plate of 20 × 32 cm. The solid medium is the conventional Youmans medium to which 10 g. Tween 80, 20 g. agar, and 7.5 mg. of malachite green per l. are added. After solidification of the agar, 48 holes are made in the plate. At least five holes are used for each dilution sample. A hole containing the standard solution at 2.5 μg./ml. of isoniazid, is alternated with a hole containing the solution under examination. The plates are kept at 37° C. for 20 hours and then the diameters of the inhibition areas are read. The best readings are between 1 and 4 μg./ml.; but 0.5 μg./ml. may often be estimated roughly.

The method described was not sufficiently sensitive for the study of the entire curve of blood levels after oral administration. Hence a dilution method was used, employing the *M. tuberculosis* H37Rv strain as

test micro-organism. The serum extracts, prepared under sterile conditions, were mixed in equal parts with Youmans medium at double the usual concentration; successive dilutions were then made with normal Youmans medium. The test-tubes were then inoculated with 0.01 mg. of the test micro-organism per ml. Controls were made by adding different amounts of the drugs to the serum extracts of untreated animals. Readings were taken after 14 days and the degree of inhibition recorded.

Chemical Determination.—The method used by us for chemical assay (Defranceschi and Zamboni, 1952) was based on the transformation of *iso*nicotinic hydrazide and its metabolic derivatives into *iso*nicotinic acid by alkaline hydrolysis in the presence of permanganate, followed by colorimetric determination of the *iso*nicotinic acid formed, by means of reaction with cyanogen bromide (Konig's pyridine nucleus reaction).

*Iso*nicotinic acid reacts with cyanogen bromide in alkaline medium, forming an intense yellow colour whose absorption maximum is at 427 mμ. The maximum depth of colour is generally obtained 15–25 minutes after addition of cyanogen bromide, when working under the best conditions, e.g., with phosphate buffer at *p*H 11, with cyanogen bromide in a final concentration of 0.5%. The reaction is performed at room temperature (20–25° C.).

The sensitivity is very high. Beer's law is followed well between 0.2 and 5 μg./ml. of final concentration in *iso*nicotinic acid (i.e., the concentration obtained after addition of reagent to the standard solutions).

Animals.—Albino rats of 170–200 g. were used. In single experiments the differences in weight did not exceed 5%. For the excretion experiments the rats were placed separately in glass metabolism cages. About half an hour before administering the drug, each animal was given 3.5 ml. of tap water by a stomach tube. Two and a half ml. more was given each time the urine was collected. The urinary bladder was expressed by massage and the cages washed thoroughly. Five rats were used for each group and their urines pooled.

When studying the blood levels or absorption via the intestine or lungs, the rats were killed at different times after administration of the drug, without giving them water. Blood was collected by puncture of the abdominal aorta, under ether anaesthesia.

A single dosage of 25 mg./kg. was used throughout all these experiments, irrespective of the route of administration. For INHM, this dose was calculated on the basis of isoniazid content of the compound. Only for paper chromatographic study of urines was 100 mg./kg. administered in order to have a sufficiently high concentration.

Results

Intraperitoneal Administration

Excretion.—Elimination in the urine was followed, beginning 30 minutes after the injection, and then every hour until the sixth hour for isoniazid and the seventh hour for INHM (Tables II and III). In these tables the rate of excretion in μg./min. during the selected time is recorded. The ratio of data from biological to that from chemical determinations allows evaluation of the extent of degradation undergone by the drugs.

Excretion reaches its maximum during the first 30 minutes after the injection and then decreases very rapidly. About 75% of the drugs administered is excreted by the end of the experiment. Both drugs behave similarly; however, when the respective rates of excretion are compared, the elimination of INHM appears to be much higher than that of isoniazid during the first hour.

Considering now the amount of active drug present in the urine, it may be seen that INHM undergoes less degradation than isoniazid. This seems not only to be related to the higher rate of excretion but to a greater stability of INHM; in fact, for this compound, some activity may still be detected seven hours after administration, whereas for isoniazid no activity is evident after five hours

TABLE II

ISONIAZID EXCRETION IN THE URINE (μG./MINUTE) AFTER ADMINISTRATION OF 25 MG./KG. BY DIFFERENT ROUTES

The data obtained by chemical and biological assays are expressed per kg. body weight

Time after Administration	Intraperitoneal		Intramuscular		Oral		Intratracheal		Intracranial	
	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.
30 min.	93·5	35·0	111·0	63·6						
1 hr.	84·0	22·0	141·0	56·0	62·0	23·6	54·0	17·7	106·0	42·2
2 ,,	84·0	9·0	107·0	21·4	142·0	25·0	80·4	12·9	110·0	22·5
3 ,,	70·0	5·0								
4 ,,	50·0	0·4	41·0	3·2	57·0	1·9	56·9	4·5	46·0	3·4
5 ,,	18·0									
6 ,,	11·0		38·0	1·4	25·0		22·3		23·2	
8 ,,			14·3	0·2	10·4		8·7		8·0	
12 ,,			2·7		3·4		3·1		3·4	
24 ,,			0·15		1·1		1·4		1·2	
% recovery ..	77·5	10·2	103·0	26·0	100·0	12·5	81·0	9·5	96·0	17·2

TABLE III

INHM EXCRETION IN THE URINE (μG./MINUTE) AFTER ADMINISTRATION OF 25 MG./KG. BY DIFFERENT ROUTES

The data obtained by chemical and biological assays are expressed per kg. body weight

Time after Administration	Intraperitoneal		Intramuscular		Oral		Intratracheal		Intracranial	
	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.
30 min.	191·0	167·0	271·0	220·0						
1 hr.	138·0	83·0	129·0	75·2	4·9	1·6	35·0	21·2	141·0	82·7
2 ,,	55·0	10·3	60·0	22·1	12·7	2·7	45·3	12·9	81·0	23·6
3 ,,	32·6	1·5								
4 ,,	31·0	0·88	30·0	4·1	21·5	1·9	52·0	5·3	40·0	14·0
5 ,,	12·0	0·40								
6 ,,	12·7	0·16	16·8	0·45	70·0	2·4	53·0	4·0	18·4	1·75
7 ,,	1·8	0·16								
8 ,,			5·0	0·10	23·9	0·3	27·2	0·9	9·4	0·83
12 ,,			1·7		9·6		13·7	0·03	3·8	0·36
24 ,,			0·4		4·5		2·6		1·5	0·03
% recovery ..	74·0	33·0	90·0	43·0	80·0	3·2	103·0	13·0	92·0	32·4

even though the total amount excreted at this time is considerable.

Blood Levels.—The blood levels are given in μg./ml. of serum. The decrease is very rapid for both isoniazid and INHM, as anticipated by the data on the rate of excretion ; the blood level of INHM decreases more promptly than that of isoniazid. No activity can be detected two hours after administration with the method used (Fig. 1).

Intramuscular Administration

Excretion.—The rate of excretion was followed from 30 minutes to 24 hours (Tables II and III). A comparison between the two drugs shows the same features as for intraperitoneal administration, that is, a higher rate of excretion and less degradation of INHM. However, if the results with both drugs are compared with the corresponding data after intraperitoneal administration, the rate of excretion appears to be greater after intramuscular administration, both for isoniazid and INHM ; furthermore, isoniazid undergoes less destruction when given this way.

Oral Administration

The drugs were administered by stomach tube in 2.0 ml. water. This route is the most interesting because it is commonly used in clinical treatment and because it has been employed by us for the assay of antituberculous activity of the drugs in mice. Therefore, the rate of absorption from the gastro-intestinal tract was investigated as well as the blood levels.

Excretion.—When compared with corresponding experiments after intraperitoneal and intramuscular injection, the results obtained (Tables II and III) show a very marked reduction of the excretion rate of both drugs. Furthermore, the excretion rates of the drugs are reversed and the excretion of

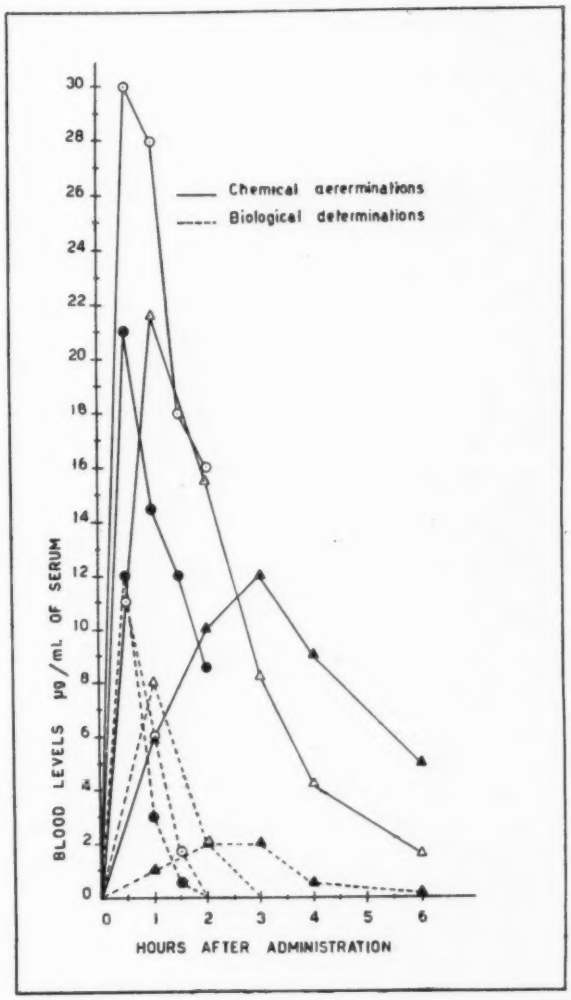


FIG. 1.—Blood levels after administration of isoniazid and INHM by different routes, in μg./ml.; chemical and biological determinations. ○ Isoniazid intraperitoneal route; △ isoniazid oral route; ● INHM intraperitoneal route; ▲ INHM oral route

isoniazid is now much more rapid than that of INHM. The data from the biological and chemical determinations must be considered separately. The chemical data show a clear displacement of the peak excretion rate from 30 minutes (parenteral administration) to the second hour for isoniazid and to the sixth hour for INHM. The peak excretion rate cannot be determined from the microbiological determinations.

For isoniazid these data are similar to those obtained after intraperitoneal injection ; for INHM they are very much lower. Some active substance is still present at the sixth hour after INHM and at the fourth hour after isoniazid. The total amount of active drug recovered after administration of INHM is lower than that after isoniazid, probably because the former remains longer within the organism.

Blood Levels.—In order to follow as completely as possible the curve of the blood levels, besides the plate method with ATCC 607, the dilution method with strain H37Rv was also used.

From both the microbiological and chemical determinations, the different behaviour of the two drugs appears very clearly. The maximum blood level of isoniazid is reached at the first hour and then decreases very rapidly, although there is a rather large amount of inactive compound still present. The levels of INHM are never so high as those of isoniazid. However, they are more constant and prolonged ; a small amount of active substance is still present after six hours, and, as seen from the chemical determinations, a maximum is reached at the third hour.

Absorption.—An analysis of the data on excretion and blood levels after administration of the two drugs by different routes induced us to believe that the differences found could only be ascribed to a different rate of absorption. Therefore, absorption from the gastro-intestinal tract was followed by determining the amount of the drugs still present, at different times after administration. The stomach and small intestine, and the large intestine, were studied separately.

A most striking difference (Tables IV and V) between the two compounds was revealed by these experiments. Isoniazid was absorbed very rapidly. During the first 30 minutes more than two-thirds disappeared from the intestine ; after four hours the drug had been almost completely absorbed before reaching the large intestine. Furthermore, the amount present in the intestine was largely inactivated. INHM, on the contrary, was absorbed at a very much slower rate ; after six hours, one-fourth of the total drug administered was still present, mostly in the large intestine. The amount of inactivated drug was also much smaller than after isoniazid ; inactivation seemed to take place more in the small than in the large intestine.

TABLE IV

PERCENTAGE RECOVERY OF ISONIAZID FROM THE INTESTINE AT DIFFERENT TIMES AFTER ORAL ADMINISTRATION OF 25 MG./KG. BODY WEIGHT

Time after Administration	Biological Assay	Chemical Assay	Biol. × 100 / Chem.
30 min.	12·2	31	39
1 hr.	8	23	35
2 ,,	4	15	27
3 ,,	1·8	7·7	23
4 ,,	1	6·6	15

TABLE V

PERCENTAGE RECOVERY OF INHM FROM THE INTESTINE AT DIFFERENT TIMES AFTER ORAL ADMINISTRATION OF 25 MG./KG. BODY WEIGHT, CALCULATED AS ISONIAZID

a = stomach + small intestine; b = large intestine

Time after Administration	Biological Assay		Chemical Assay		Biol. × 100 / Chem.
30 min. a	96		93		103
1 hr. a	77		90		85
2 ,, a	34	51·6	45·3	69·3	75
2 ,, b	17·6		24		73
3 ,, a	6·4	40·8	13	59·5	49
3 ,, b	36·4		46·5		78
4 ,, a	5·5	5·7	11·2	45·7	49
4 ,, b	20·2		34·5		59·5
6 ,, a	—		5·1	24·1	
6 ,, b	13·8		19		72·5

An apparent discrepancy arises when comparing the inactivation rate of the two drugs within the intestine and the respective amounts of active substance found in the urine. This may easily be explained by the rapid absorption and, consequently, rapid excretion of isoniazid before inactivation could be completed by the organism ; in fact *in vitro* studies (De Carneri, 1952, unpublished) show that the time required for inactivation is rather long even in organs as active as liver, at optimal concentrations.

Intratracheal Instillation

Administration by this route was studied for two reasons ; firstly, to see if absorption through the bronchial mucosa, like the intestinal, could also reveal differences of behaviour between isoniazid and INHM and, secondly, because it would be interesting, from a practical point of view, to see whether it was possible to obtain a high concentration of these two drugs in the lungs, which was unobtainable by other routes.

Direct instillation was preferred to administration by aerosol in order to have more accurate

control of the amount introduced. Therefore, after incision of the skin of the throat under local anaesthesia, the salivary glands were separated in the mid line and the trachea reached through the pretracheal muscles and freed for about half a centimetre. The solution (0.1 ml.) was then instilled slowly into the tracheal lumen, using a very fine needle.

Excretion.—The results (Tables II and III), are very similar to those obtained by oral administration. The excretion peak, as seen from the data on chemical determinations, is reached at the fourth hour after isoniazid and at the sixth hour after INHM. However, when the results of the microbiological determinations are considered, the differences between the two drugs are not so striking. In fact, after both isoniazid and INHM, the maximum amount of active substance is excreted in the urine during the first hour. The difference between isoniazid and INHM consists chiefly in the fact that the active substance is excreted in higher quantity and over a longer period after the latter drug.

Absorption—(Table VI).—Absorption is very rapid. After isoniazid the drug cannot be detected in the lungs as early as 30 minutes after instillation. INHM is present in active form until the second hour but only to about 1% of the amount injected. However, if the ratio of drug to weight of pulmonary tissue is examined, the concentration appears to be high, and worth considering for therapeutic purposes.

Intracranial Administration

Intracranial administration is also interesting both from a theoretical and a practical point of view. It would, in fact, be worth knowing whether the meninges behave similarly to the intestinal and bronchial or to the peritoneal membrane. Furthermore, as streptomycin is usually preferred to dihydrostreptomycin in the treatment of meningeal tuberculosis, and its toxicity is increased by isoniazid, though not by INHM (Ceriotti, 1953, c and d), it was important to learn whether the latter could be advantageously substituted for the former.

The drugs were injected intracranially in 0.05 ml. of solution by the transorbital route. A short period of convulsions sometimes followed the injection ; the animals, however, usually showed no important disturbances. Only the excretion was studied.

Excretion.—Excretion after intracranial administration (Tables II and III) does not show great differences between isoniazid and INHM. The maximum is attained during the first hour after INHM, a little later after isoniazid ; there is then a sharp decrease in the excretion rate. However, it may be also observed here that the degradation of INHM proceeds at a lower rate than that of isoniazid. In fact, some active substance is still detectable in the urine at the twelfth and sometimes at the twenty-fourth hour after INHM administration ; after isoniazid on the other hand no activity can be found after the fourth hour.

Chromatographic Analysis

In order to learn how isoniazid and INHM were degraded during their passage through the body, a paper chromatographic method for examination of the urine was devised ; a more detailed description will be published elsewhere (Defranceschi, 1953). The dose of both compounds administered during this experiment was 100 mg./kg. intraperitoneally in order to give rather high concentrations in the urine.

The urine content of isoniazid and INHM was determined both chemically and microbiologically, and the concentration was then adjusted so as to give 20 μg. in 10 μl. as determined chemically. This was not possible for urine samples collected after 120 minutes, because they were already too diluted ; a larger amount of urine was therefore used without further dilution. Standard solutions

TABLE VI

ISONIAZID AND INHM, RECOVERY FROM LUNGS AT DIFFERENT TIMES AFTER INTRATRACHEAL INSTILLATION OF 25 MG./KG. BODY WEIGHT

Time after Administration	Isoniazid				INHM				
	Biological		Chemical		Biological		Chemical		Biol. × 100 / Chem.
	mg.	μg./g. of Lung	mg.	% of Drug Administered	mg.	μg./g. of Lung	mg.	% of Drug Administered	
30 min.	—	—	2·25	0·9	0·260	52·0	0·385	1·5	68
1 hr.					0·164	32·8	0·328	1·3	50
2 ,,					0·168	33·6	0·328	1·3	51
3 ,,							0·236	0·94	
4 ,,							0·215	0·86	

containing 20, 10, and 5 μg. in 10 μl., were developed at the same time. Several samples of each urine were chromatographed.

Strips of Whatman paper No. 1 (1.5 × 37 cm.) were used. The urine samples were applied 6 cm. from the edge, which was immersed in the solvent. Irrigation was carried out with the following solvent mixture: isoamyl alcohol (50), water (50), acetic acid (1.5). Only the organic (upper) layer was used. After development, the spots were detected both chemically and biologically.

For chemical detection the dried paper strips were suspended in a closed glass jar, saturated with cyanogen bromide vapours. After 20 minutes, 20 ml. of 3% aqueous ammonia was poured out on the bottom of the jar. After another 15 minutes, pyridine derivatives (namely, *iso*nicotinic acid, nicotinic acid, nicotinamide) gave an intense yellow colour; isoniazid and INHM gave brownish-yellow spots and were better detected under ultra-violet light (mineral light lamp), giving an intense bluish fluorescence. The Rf found after chromatography with the solvent mixture used are as follows: isoniazid (0.80), INHM (0.01), *iso*nicotinic acid (0.42), nicotinic acid (0.51), nicotinamide (0.48), and compound X (0.26).

The intensity of single spots could be estimated roughly by this method and microbiologically-inert compounds, containing the pyridine group, detected. More quantitative evaluation was obtained by the biological method, which estimates the active material. After development and drying, the strips were sterilized by ethylene oxide, in order to avoid contamination. They were then placed on large agar plates, inoculated with ATCC 607, as for the cup method. After 23 hours of incubation at 37° C., the diameters of the inhibition were recorded and the areas calculated by the ellipse formula. The areas of inhibition of standard samples, when plotted against the logarithm of the concentration, gave a straight line, which was taken as a basis for calculating the concentration of the active substances in samples of urine (Fig. 2).

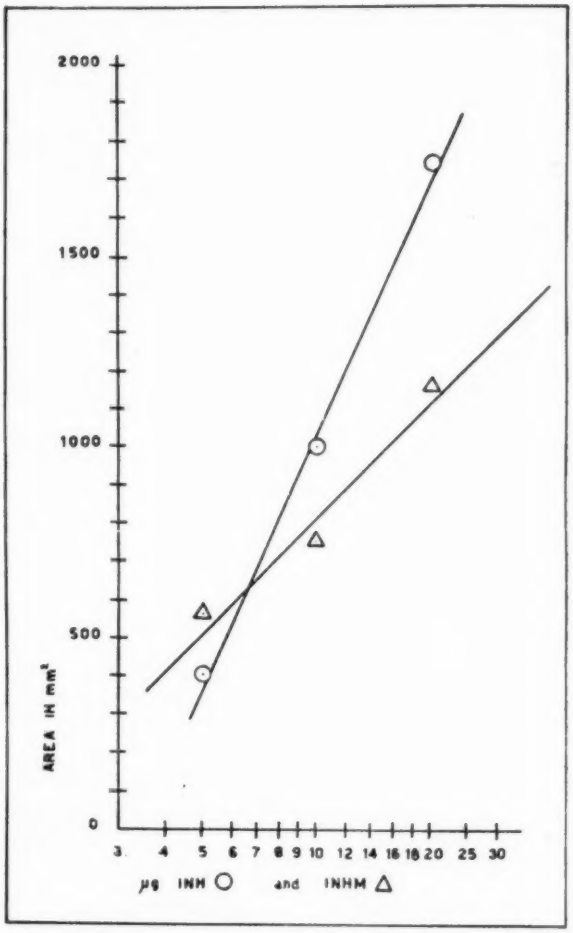


FIG. 2.—Filter paper chromatography of isoniazid and INHM. Detection on germ-agar plate (ATCC 607). Inhibition areas plotted against the log of the amount of drugs in μg.

This method is not strictly quantitative because the variations are still rather large. However, it allows a good semi-quantitative estimation of the amounts of active substances present. Furthermore, separation and identification of different active compounds is made possible. This is particularly important here, because it demonstrates that INHM gives rise to a moderate amount of isoniazid. The results obtained both by chemical and microbiological detection are summarized in Table VII. As may be seen from these data, the

TABLE VII

PAPER CHROMATOGRAPHY EXPERIMENTS ON URINES OF RATS, GIVEN 100 MG./KG. OF ISONIAZID AND INHM INTRAPERITONEALLY

The percentages of the biologically active substance found by direct micro-determination and after chromatography are compared

Time after Injection (min.)	Isoniazid				INHM				
	Micro-deter.	Chromatographic Data			Micro-deter.	Chromatographic Data			
	Biological Assay	Biological Assay	Isonicotinic Acid	Compound X	Biological Assay	Biological Assay INHM	As Isoniazid	Isonicotinic Acid	Compound X
30	94	80	+++	−	82	105	−	±	−
60	65	71	++++	+	64	94	±	++	±
120	43	40	++++	++	42	41	24	+++	++

percentages of biologically-active substance determined by chromatographic and plate-cup method agree rather well for isoniazid. For INHM, however, the plate gives consistently lower values. These results agree with those of the recovery experiments already discussed ; it seems that urine contains a substance or substances which inhibit the action of INHM more than that of isoniazid, and are dissociated from the active compound by the chromatographic procedure. *Iso*nicotinic acid is formed from both compounds, but at a much higher rate from isoniazid. Another not yet identified compound, which reacts with CNBr and has a lower Rf than *iso*nicotinic acid, is released more promptly by isoniazid. In conclusion, the paper chromatographic experiments show a more rapid degradation of isoniazid as compared with INHM ; some isoniazid is also formed from the latter.

Determination of Ionization Constants

Although the difference in the degree of dissociation of isoniazid and INHM arises directly from a consideration of their formulae, and needs no special demonstration, yet to obtain more detailed information and quantitative data, the ionization constants of the two compounds were determined. The determination for INHM was made by direct titration ; for isoniazid the spectrophotometrical method had to be used instead. Several related compounds were also studied by this method, and the results will be referred to elsewhere. For the SO_3 group of INHM, the pK_a was found to be 3.75 ; for isoniazid the hydrazine group gave a value of $pK_{b_1} = 10.3$, and the pyridine nitrogen of $pK_{b_2} = 12.2$.

It can be stated on the basis of these results that, at physiological *p*H, INHM is completely dissociated and isoniazid completely undissociated.

Discussion

Before discussing the results obtained, the methods of determination deserve some consideration. Recovery is practically complete, both by microbiological and by chemical determination, so far as intestine and lungs are concerned.

From serum, only 80% recovery is possible microbiologically. From urine isoniazid is recovered almost completely when urine samples are diluted 1 to 10 ; but INHM is recovered only partially even at a dilution of 1 to 200. This phenomenon may be explained by assuming that some substances which inhibit INHM are present in urine, and that these substances have only a very low inhibitory action towards isoniazid. This supposition is confirmed when the results obtained by the cup-plate method are compared with the microbiological data from chromatographic experiments. The ratio of biologically-active substances to the total determined chemically is much higher after chromatography. This means that the supposed antagonist substance or substances move along the paper strips at a different rate to INHM and the latter is therefore no longer inhibited and can exert its action completely. These facts suggest that the mode of action of the two substances may be partly different. This conclusion is supported also by the observation (Ceriotti, 1953b) that an ATCC 607 strain, made dependent upon INHM, does not grow in the presence of isoniazid. The difference is only partial, because some isoniazid is recovered in urine after the administration of INHM ; and this means that the latter certainly acts also through its degradation to isoniazid. The biological problem arising from the preceding results, and involving the mode of action of the two drugs, is now under examination.

Although the interference of unknown inhibitory substances does not allow an absolute evaluation of isoniazid by the microbiological cup-plate method in certain experiments, this does not seem to alter the general conclusions drawn from the results obtained, as far as the problems under study are concerned. Some of these conclusions may also be emphasized. In fact, a phenomenon generally observed in the majority of our experiments was the lower degradation rate of INHM as compared with isoniazid. This phenomenon is partially concealed by the antagonistic action of the urine, and it appears even more evident when the latter is excluded by chromatography. It must therefore be concluded that the difference in degradation rate between isoniazid and INHM is also greater than that one disclosed by the microbiological determinations. The lower degradation rate of isoniazid may be seen clearly from the results of experiments on absorption from the intestine as well as from the excretion data. In these experiments, the values from the microbiological cup-plate method can also be considered absolute, as shown by the recovery experiments. Isoniazid appears to be broken down considerably in the intestine before being absorbed, whereas INHM remains unaltered for a fairly long time.

The experiments with oral administration received particular attention because they are more intimately connected with *in vivo* tests for activity ; they were conducted by the diet–drug method. When the two drugs are administered by mouth, besides being rather rapidly destroyed, isoniazid is also absorbed at a much higher rate

than INHM and is therefore excreted more quickly. The blood levels attain a higher maximum with isoniazid, but they also fall rapidly. With INHM, on the contrary, as a result of the slow rate of absorption, the blood levels are not so high, but are maintained longer. Owing to the extreme sensitivity of *M. tuberculosis* to these drugs, this behaviour seems to be more favourable to therapeutic action. In fact, if the concentration required for drug activity is low, it is conceivable that high, but transient, levels of drug concentration exert a lesser effect than low, but stable levels, provided that the latter exceeds the minimum required for effective action. Beyond these limits an increase in concentration may not be paralleled by a corresponding increase in activity. Both the lower degradation rate and slower absorption and, consequently, the more prolonged blood levels of INHM, administered orally, may account for the better protection afforded by this drug in experimental mouse tuberculosis.

Another important difference between isoniazid and INHM may be observed in relation to the various routes of administration. The data of excretion rates, as summarized in Figs. 3 and 4, show this difference clearly. After administration of isoniazid, the excretion maxima lie between half an hour for intraperitoneal, and two hours for oral administration. With INHM, on the other hand,

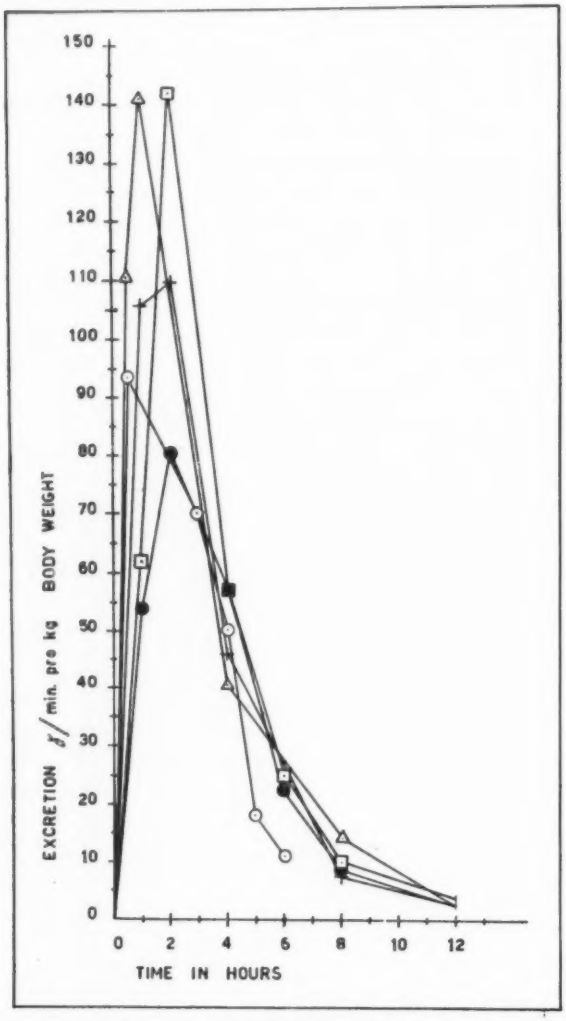


FIG. 3.—Excretion curves of isoniazid after administration by different routes. Results of chemical determinations in μg. per min. per kg. body weight. ○ Intraperitoneal route; △ intramuscular route; □ oral route; ● intratracheal route; × intracranial route.

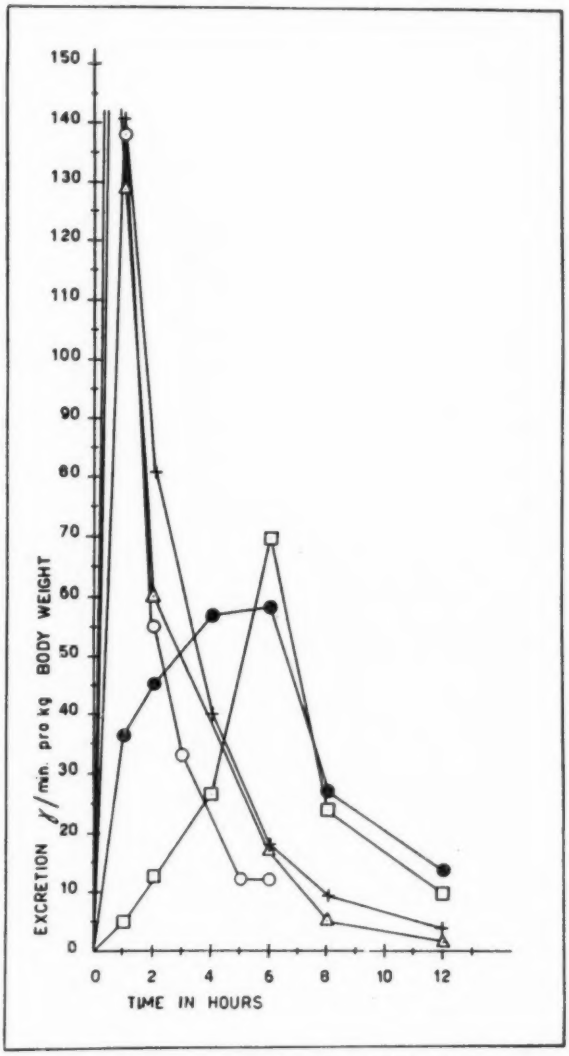


FIG. 4.—Excretion curves of INHM after administration by different routes. Results of chemical determinations in μg. per min. per kg. body weight. ○ Intraperitoneal route; △ intramuscular route; □ oral route; ● intratracheal route; × intracranial route.

there is a much larger variation. After intraperitoneal and intramuscular administration, excretion is almost immediate and reaches a very high peak after half an hour. After oral and intratracheal administration the maximum is shifted towards the sixth hour. These results are to be correlated with the different rates of absorption. For isoniazid, passage through the intestinal mucosa does not differ much from passage through the peritoneal serosa or the capillary walls, whereas INHM passes slowly through the mucosae. Recovery from the intestine gives direct evidence of this assumption, as does that from lungs, though this is not so striking.

The physico-chemical properties of the two drugs may supply some explanation of these findings. Isoniazid is soluble both in water and in organic solvents. INHM as the Na or Ca salt is soluble practically only in water ; at *p*H 7–8, the former is completely undissociated, and the latter completely dissociated. It is well known that substances which are soluble both in water and organic solvents generally penetrate the intestinal wall most rapidly ; moreover, the rate of absorption is dependent on the degree of dissociation, since it is higher for less dissociated compounds, as has been demonstrated for alkaloids and a large series of sulphonamide drugs (Travell, 1940 ; Ellison, and Richardson, 1938 ; Poth and Knotts, 1942 ; Poth, Knotts, Lee, and Jami, 1942 ; Fisher, Troast, Waterhouse, and Shamon, 1943). The rate of excretion, however, does not show a similar relationship (Fisher, Troast, Waterhouse, and Shamon, 1943). The present experiments agree well with the observations on sulphonamides, in so far as absorption through the intestinal wall is concerned. However, INHM readily crosses the blood–brain barrier, as shown by the results of intracranial administration.

In summing up the experiments described in this paper, some differences between isoniazid and INHM may be pointed out: INHM is absorbed at a much lower rate than isoniazid through the intestinal wall ; it is also much less degraded and thus creates more stable active blood levels. This behaviour finds its basis in the physico-chemical properties of the two drugs. INHM seems to act both directly and through its transformation to isoniazid.

Summary

1. Absorption, blood levels and excretion rate were studied after administration of *iso*nicotinic acid hydrazide (isoniazid) and of sodium and calcium salts of N′-*iso*nicotinoylhydrazinomethanesulphonic acid (INHM) by different routes.

2. Degradation products were detected in the urine by paper chromatography.

3. Some physico-chemical properties of the two drugs were determined.

4. No great difference between isoniazid and INHM could be observed when intraperitoneal, intramuscular, or intracranial routes of administration were used. However, the highly-dissociated INHM was absorbed slowly through the intestinal wall. Absorption and excretion were also less after intratracheal administration and INHM was degraded at a lower rate than isoniazid.

5. *Iso*nicotinic acid and an unknown compound, reacting with BrCN, are formed both from isoniazid and INHM ; however, this formation is less rapid after INHM, which also gives rise to some isoniazid.

We are indebted to Miss Emma Berti, Messrs. Pio Gagliardi, Franco Borghi, and Mario Grechi, for their kind technical assistance in the present work.

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TISSUE DISTRIBUTION OF α-^{14}C-LABELLED ALKYLOXY α-PHENYLETHYLAMINES IN RELATION TO ANALGESIC ACTIVITY

BY

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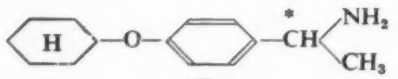
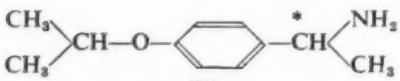
(RECEIVED APRIL 18, 1953)

Minor changes in chemical structure could influence the physiological response to a drug by causing changes in those physical properties which determine access to susceptible tissues. The tissue distributions of ^{14}C-labelled *p-cyclo*hexyloxy-α-phenylethylamine (I) and *p-iso*propyloxy-α-phenylethylamine (II) have been studied with this concept in mind, since I has morphine-like properties not possessed by II (McCoubrey, 1953). The α-C-atoms (marked with an asterisk in formulae I and II) were the labelled atoms.



(I)



(II)

METHODS

The amines were given as the hydrochlorides in water. The doses (40 mg./kg. i.p. and 10 mg./kg. i.v.) were sufficient for I, but not for II, to induce well-marked analgesia (M.E.D. 10–15 mg./kg. i.p.). Animals were killed by excision of the heart under ether anaesthesia (3 minutes) unless stated otherwise. Tissues were always dissected in the same order. There was little change in tissue concentration during thirty minutes after exsanguination (Brierley, unpublished). Visceral tissues were removed rapidly after intraperitoneal injection to avoid an observed *post-mortem* absorption of drug from the peritoneal cavity. To obtain sufficient tissue, six male albino rats (300–350 g.) were used for each time interval. Approximately equal amounts of any one tissue were taken from each animal and pooled. Rabbits of one litter were used for each drug, two animals per time interval. The analgesia due to I was sufficient to allow killing, without anaesthesia, by medullary section through the atlanto-occipital membrane, sometimes after withdrawal of cerebrospinal fluid. Ether was required for II. The monkey (*Macaca mulatta*, ♂, 3.5 years) was lightly anaesthetized (ethyl chloride, then ether) before giving I into the saphenous vein.

Definition of Tissues.—Meninges and vessels were removed as completely as possible. *Cerebral cortex*—thin slices excluding visible white matter. *Subcortical white matter*—substantial contamination with grey matter is inevitable in small animals. Monkey corpus callosum is essentially pure white matter. *Thalamus*—isolated by coronal cuts at the interventricular foramen and habenula, laterally by an oblique cut at the junction with the internal capsule, inferiorly at the hypothalamic sulcus. *Hypothalamus*—from optic chiasma anteriorly to the mammillary bodies posteriorly and extending superiorly to the hypothalamic sulcus. *Cerebellum*—midline vermis with white core removed. *Medulla*—from auditory tubercle to obex with removal of area postrema. *Spinal cord*—cervical or lumbo-sacral enlargements. In certain cases cervical cord grey matter was carefully dissected from the white matter. *Dorsal root ganglia*—lumbo-sacral and cervical ganglia were pooled from rats, but were treated as separate groups in rabbit and monkey. The ventral roots were removed. Peripheral nerve and dorsal root were removed as close to the ganglion as possible. *Sympathetic ganglia*—superior cervical and stellate ganglia. *Adrenals*—rat adrenals were decapsulated. Rabbit adrenals were cut in three. The two outer thirds ("cortex") were assayed separately from the inner third.

Radioassay.—All tissues were dried at 60° C. 50 or 30 mg. of powdered tissue were assayed as "infinite thickness" samples (Popják, 1950) under a G.E.C. EHM2 mica window (1.9 mg./cm.^{2}) counter. The counting efficiency by comparison with a standard ^{14}C-polythene disc was 1.9%. Where insufficient dry tissue was available, kieselguhr was added and thoroughly mixed to make 30 mg. A linear relation between such dilution and activity was found in trial assays with various tissues. 2,000 counts, including background (10–13 counts per minute), were recorded for most tissues (error not greater than ±4.5%). 1,000–1,500 counts were recorded for tissues with low activity

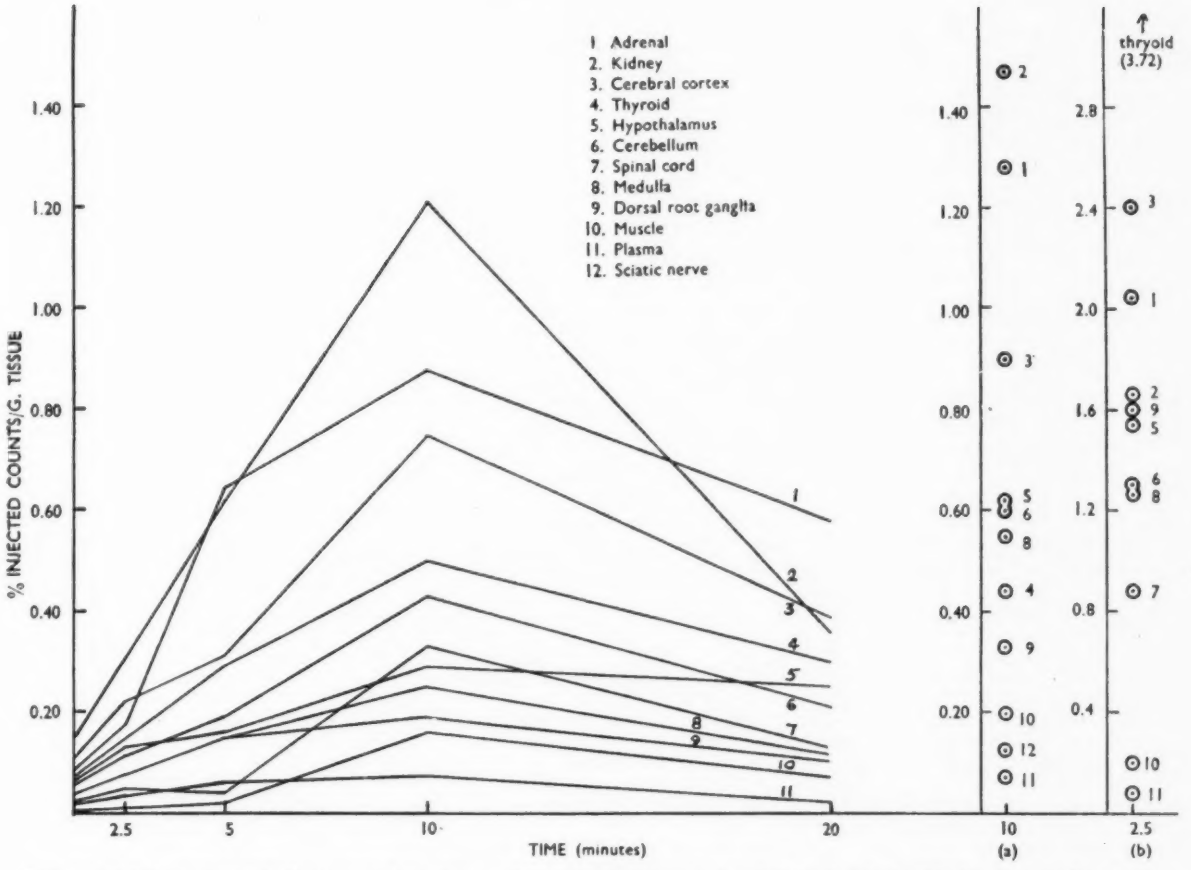


FIG. 1.—Rat tissue concentrations at various times after *p-cyclo*hexyloxy-α-phenylethylamine hydrochloride (40 mg./kg. i.p.). The ordinates represent percentage of injected counts per animal per gramme wet tissue. (*a*) Gives the tissue activities of *p-iso*-propyloxy-α-phenylethylamine under comparable conditions at ten minutes. (*b*) Gives the tissue activities at 2½ minutes after the *cyclo*hexyl ether (10 mg./kg. i.v.).

error not greater than ±13%). Tissue activities were calculated as percentage of injected counts per animal per g. wet tissue.

RESULTS

Tissue concentrations of I and II in the rat at various times after 40 mg./kg. intraperitoneally are given in Fig. 1, together with the concentrations of I at 2½ minutes after 10 mg./kg. intravenously. Results for the rabbits, which received 10 mg./kg. of I or II intravenously, are given in Fig. 2. These results are for one of two rabbits per time interval except where pooling of tissue was necessary. No significant difference in important tissues was observed in such pairs. Table I gives the concentration of I in monkey tissue five minutes after receiving 10 mg./kg. intravenously.

There was only low activity in muscle, liver, sciatic nerve, testis, trigeminal ganglion, and pituitary in both rat and rabbit. There was no activity in cerebrospinal fluid.

TABLE I

RADIOACTIVITY OF MONKEY TISSUES FIVE MINUTES AFTER *p-CYCLO*HEXYLOXY-α-PHENYLETHYLAMINE HYDROCHLORIDE (10 mg./kg.) INTRAVENOUSLY

Activity is expressed as the percentage of injected counts per gramme wet tissue.

Tissue	Activity	Tissue	Activity
Sensory cortex ..	0·109	Dorsal nerve root	0·016
Motor ,, ..	0·113	Dorsal root ganglion (cervical)	0·029
Visual ,, ..	0·110	Dorsal root ganglion (lumbar)	0·040
Frontal ,, ..	0·109	Dorsal root ganglion (sacral)	0·029
Cerebellar ,, ..	0·099	Sympathetic ganglion	0·075
Olfactory lobe ..	0·064	Sciatic nerve	0·006
Ventral thalamus ..	0·113	Saphenous nerve	0·013
Dorsal thalamus ..	0·095	Kidney cortex	0·338
Hypothalamus ..	0·062	Adrenal	0·333
Medulla	0·076	Pituitary	0·193
Corpus callosum ..	0·012	Thyroid	0·068
Spinal cord		Muscle	0·016
,, ,, (lumbar)	0·062	Liver	0·020
Spinal cord (cervical grey matter) ..	0·100	Whole blood	0·0047
Spinal cord (cervical white matter) ..	0·018	Plasma	0·0060

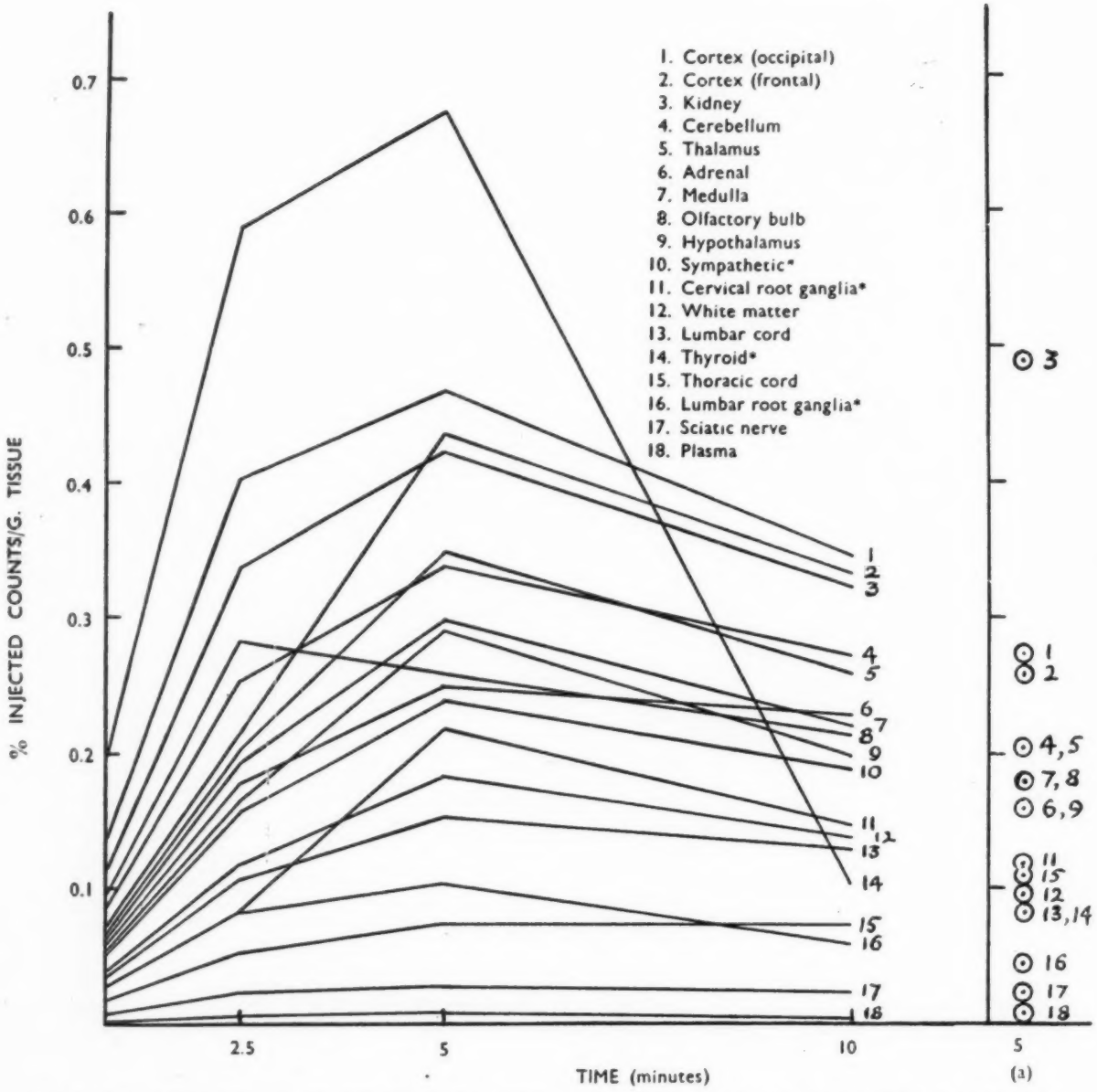


FIG. 2.—Rabbit tissue concentrations at various times after *p-cyclo*hexyloxy-α-phenylethylamine hydrochloride (10 mg./kg. i.v.). * Indicates where tissues were pooled from two animals. (*a*) Gives the tissue activities of *p-iso*propyloxy-α-phenylethylamine under comparable conditions at five minutes.

DISCUSSION

I and II, like most basic substances, readily penetrate nervous tissues (Friedemann, 1942). Table II clearly shows that pharmacological differences between these amines cannot be explained in terms of access to tissues. These amines have no markedly different preferences for nervous tissues, and II, which is not analgesic in the rat, penetrates all rat tissues examined more readily than I. The converse applies to the rabbit, but no pharmacological data with respect to these substances are available for this species. The tissue concentration-time curve (Fig. 1) is similar to the analgesic time-action curve for I in the rat and does not correspond to the time course of respiratory depression (McCoubrey, 1953). The ephemeral action of I is probably due to rapid elimination by the kidney. It was not possible to show any marked plasma activity, even after intravenous dosage, and the amines appear to be rapidly con-

TABLE II

COMPARATIVE TISSUE CONCENTRATIONS OF *p-CYCLO*HEXYLOXY (I) AND *p-ISO*PROPYLOXY-α-PHENYLETHYLAMINE (II) IN RAT AND RABBIT

Activities are calculated from $\frac{a \times n}{p \times N}$ where a = counts per gramme wet tissue, n = number of animals, p = counts per mil. in plasma, N = total injected counts for the group. The plasma activities (% injected counts per animal per gramme wet tissue) were respectively: rat: I, 0.071, II, 0.075; rabbit: I, 0.010, II, 0.013

Tissue	Rat			Rabbit		
	10 min. after 40 mg./kg. i.p.			5 min. after 10 mg./kg. i.v.		
	I	II	Ratio	I	II	Ratio
Cerebral cortex ..	10·6	13·5	1·27	—	—	—
,, occipital ..	—	—	—	47·0	20·6	0·45
,, frontal ..	—	—	—	43·4	20·4	0·47
Hypothalamus ..	4·2	8·2	1·95	29·8	12·4	0·42
Thalamus	—	—	—	34·6	15·6	0·45
Cerebellum	6·1	8·0	1·31	33·8	15·6	0·46
Medulla	3·5	7·3	2·10	30·0	13·8	0·46
Olfactory bulb ..	—	—	—	25·0	13·9	0·56
Dorsal root ganglia ..	2·8	4·4	1·58	—	—	—
,, lumbo-sacral ..	—	—	—	10·5	3·5	0·38
,, cervical ..	—	—	—	15·6	6·6	0·42
Sympathetic ganglia ..	—	—	—	24·2	6·6	0·27
Adrenal	12·4	17·0	1·37	23·2	12·4	0·54
Thyroid	7·0	5·9	0·84	67·5	8·8	0·13
Kidney	17·1	19·6	1·15	42·5	38·0	0·89
Muscle	2·2	2·6	1·18	6·8	4·4	0·65

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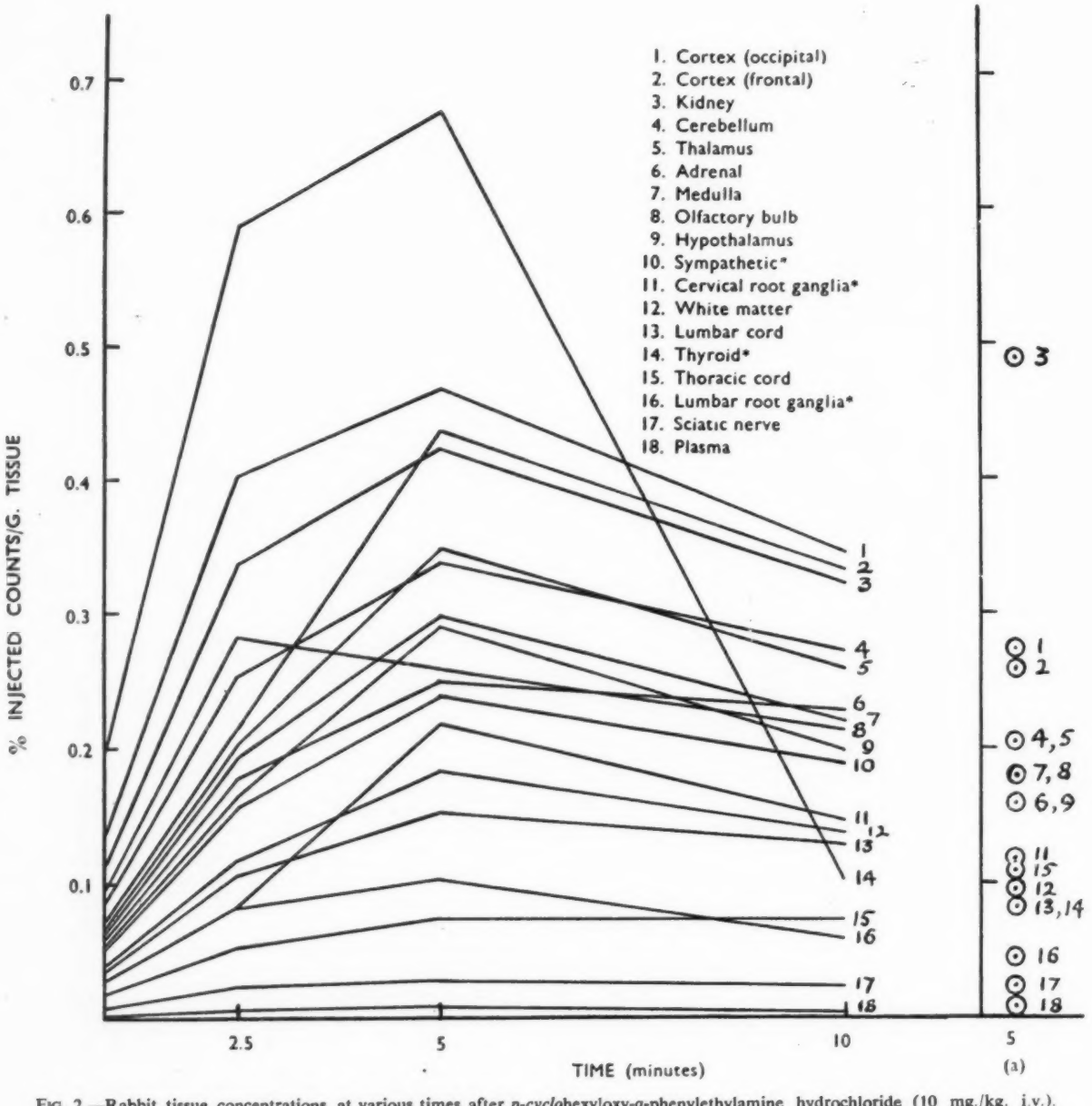
It is not possible at present to relate the selective concentration in rat or rabbit cerebral cortex to analgesic activity. Similar selective localization does not occur in the monkey and in this animal there is no preference for either sensory or frontal cortex. There is little evidence that pain sensation is normally projected on to the cerebral cortex even in man. Penfield (1947) has shown that electrical stimulation of exposed human cerebral cortex in conscious subjects evokes no painful sensation, and, while section of frontal lobe tracts (lobotomy) relieves the anxiety associated with pain, it apparently does not reduce pain sensation, and indeed the threshold for experimental pain may be reduced (Chapman, Rose, and Solomon, 1948). In isolated cases excision of sensory cortex can relieve pain of long standing (Lewin and Phillips, 1952), but in cases of intractable pain the most effective procedure appears to be section of spinothalamic tracts (Falconer, 1953).

There are indications that some, but certainly not all, aspects of analgesic mechanism are mediated at a spinal level. Spinal structures were therefore examined in some detail, especially the dorsal root ganglia, since all afferent sensory impulses traverse these ganglia. Brierley (1951, 1952) has shown that they concentrate a number of ions, and, although there are no synapses here, a brief delay occurs in impulse transmission across the ganglia (Dun, 1951), which could indicate actual participation by the cells in transmission. In the dorsal horn grey matter, Pearson (1952) finds histologically that slow pain fibres make their synapse in the dense neuropil of the *substantia gelatinosa Rolandi*, whereas fast pain fibres by-pass this region to synapse in the *nucleus proprius*. This is of interest in view of Cushny's remark (1941) that "sudden shocks" (fast pain?) are distinguishable under morphine while pain (slow pain?) is under control. Further, spinal animals react equally well in analgesic assays which measure depression of avoidance reflexes (Bonnycastle and Leonard, 1950; Houde and Wikler, 1951). The appreciable concentration of drug, however (approx. 10^{-7} moles per g. tissue), which appeared at all cell stations on the pain pathway, viz., dorsal root ganglia, spinal cord grey matter, medulla and thalamus, with little in the interconnecting fibre tracts and peripheral nerve, suggests that the action of analgesics may be an attenuation of pain impulses at the successive cell stations.

The difference in pharmacological response between I and II had suggested that some differences in localization might occur in the nervous system. Marked divergence was only observed in the adrenal and thyroid glands. Mere concentration does not necessarily imply a role for these glands in analgesia; there is considerable evidence that their secretions markedly influence the action of analgesics, but discussion can only be speculative in the absence of experimental results.

CHEMICAL SECTION

The route previously used for synthesis of alkyloxy-α-phenylethylamines was not wholly suitable for ^{14}C-labelling. *p-Cyclo*hexyloxyphenyl magnesium bromide was carbonated to give the carboxylic acid, whence the lithium salt and lithium methyl (Gilman and Van Ess, 1933) gave *p-cyclo*hexyloxyacetophenone. The ketoxime was reduced to the amine as previously described (McCoubrey and Iyengar, 1951). The *iso*propyl ether was prepared similarly.



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p-*Bromophenyl* Cyclo*hexyl Ether*.—*p*-Bromophenol (20 g.) and *cyclo*hexyl bromide (57 g.) by the method described (McCoubrey and Iyengar, 1951) gave the *ether* (10.9 g.), b.p. 115–117°/0.8 mm. It crystallized from light petroleum in prisms m.p. 40–41°. (Found: Br, 31.3. $C_{12}H_{15}OBr$ requires Br, 31.4%.)

p-*Bromophenyl* Iso*propyl Ether*.—Prepared conventionally. The *ether* was a fragrant oil, b.p. 119–120°/20 mm., $n_D^{20°}$, 1.5368. It could be crystallized from light petroleum m.p. 17.5°. (Found: C, 51.4; H, 4.2; Br, 36.5. $C_9H_{11}OBr$ requires C, 50.2; H, 5.1; Br, 37.2%.)

p-Cyclo*hexyloxy- and* p-Iso*propyloxybenzoic Acid*.—The above bromides were converted to Grignard reagents by boiling with magnesium and a few drops of ethyl iodide in ether under nitrogen for five hours. The filtered solutions were carbonated in conventional manner with carbon dioxide generated from barium carbonate. The products were isolated conventionally. The *cyclo*hexyl ether (77%) crystallized from ethanol in plates m.p. 176°. (Found: C, 70.7; H, 7.2. $C_{13}H_{16}O_3$ requires C, 70.1; H, 7.3%.) The *iso*propyl ether (72%) crystallized from ethanol in plates m.p. 159° (softens at 140°). (Found: C, 66.1; H, 6.4. $C_{10}H_{12}O_3$ requires C, 66.7; H, 6.7%.)

The acids were dissolved in ether and were treated cautiously with three molecular proportions of lithium methyl and boiled overnight. The acetophenones were isolated, converted to the ketoximes and reduced by sodium amalgam to the amines.

α-^{14}C-labelled *p-cyclo*hexyloxy-α-phenylethylamine was obtained in 40% overall yield (1.28 g.) with an activity of 8,296 counts per minute per mg. α-^{14}C-labelled *p-iso*propyloxy-α-phenylethylamine was obtained in 40% overall yield (0.95 g.) with an activity of 10,562 counts per minute per mg. Yields are calculated on barium carbonate used.

Summary

*p-Cyclo*hexyloxy- and *p-iso*propyloxy-α-phenylethylamine hydrochlorides penetrated with equal facility into rat, rabbit, or monkey cerebral grey matter. A selective localization in rat and rabbit cerebral cortex cannot at present be correlated with analgesic mechanism. There was no localization in peripheral nerve.

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